SENSIBILIDAD DE ESPECIES PASCÍCOLAS MEDITERRÁNEAS ANUALES AL INCREMENTO DE OZONO TROPOSFÉRICO: PARÁMETROS DE RESPUESTA Y DEFINICIÓN DE NIVELES CRÍTICOS

JAVIER SANZ GONZÁLEZ

Madrid, 2017

TESIS DOCTORAL

UAM
UNIVERSIDAD AUTONOMA
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GOBIERNO DE ESPAÑA
MINISTERIO DE ECONOMÍA, INDUSTRIA Y COMPETITIVIDAD

Ciemat
Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas

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HACEN CONSTAR QUE: El presente trabajo titulado “Sensibilidad de especies pascícolas mediterráneas anuales al incremento de ozono troposférico: parámetros de respuesta y definición de niveles críticos” ha sido realizado por el Licenciado Don Javier Sanz González bajo nuestra dirección en el Grupo de Ecotoxicología de la Contaminación Atmosférica, del Departamento de Medio Ambiente, del Centro de Investigaciones Energéticas Medioambientales y Tecnológicas (CIEMAT), constituyendo la Tesis Doctoral de su autor.

Madrid, 12 de enero de 2017

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AGRADECIMIENTOS

Como no puede ser de otra manera, tengo que comenzar esta ronda de agradecimientos recordando a quien un día me dio su confianza, y la razón por la cual yo le estaré agradecido todos los días, ¡muchas gracias Benja!, ¡muchas gracias jefe! ¡te debo mucho amigo! Continuo con una de las directoras, bueno, en este momento también la jefa, que desde luego sin su apoyo, su paciencia, y por supuesto su trabajo y esfuerzo, no me hubiera sido posible concluir este trabajo, ¡muchas gracias Viki! Y termino esta primera remesa con el compañero con el que pase tantos días, tantos meses, durante cuatro años, en el campo experimental del Delta del Ebro, ¡muchas gracias Mendoza!, fue todo un placer trabajar contigo amigo.

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PROLOGO

Hasta mediados del siglo pasado, fue prácticamente inexistente el interés de los ciudadanos y de las instituciones gubernamentales hacia los problemas generados por su propia actividad y modelo de desarrollo. Desde entonces se ha ido incrementando la sensibilidad colectiva a medida que los problemas ambientales se han hecho más perceptibles, y las evidencias científicas han puesto de manifiesto el peligroso camino de desarrollo actual que lleva a la Tierra a un punto sin retorno. Así, la contaminación atmosférica y sus efectos en la vegetación, la salud humana y el medio ambiente en general, son un campo de actuación prioritaria en los programas de investigación y en las políticas en materia de protección ambiental a escala mundial.

Durante la última década, el Grupo de Ecotoxicología, del Departamento de Medio Ambiente, del CIEMAT (Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas) ha desarrollado una línea de investigación sobre el efecto del ozono troposférico en especies herbáceas anuales características de las comunidades pascícolas de la dehesa, un ecosistema de un gran valor ambiental y un ejemplo de sostenibilidad, protegido por la Directiva Hábitat 92/43/EEC e incluido en la red Natura 2000. La base experimental y el conocimiento adquirido mediante estos estudios son la base de la presente memoria.

Los estudios presentados han sido realizados en el marco del Convenio de Naciones Unidas sobre Transporte Transfronterizo a Gran Distancia de Contaminantes Atmosféricos (*Convention on Long-Range Transboundary Air Pollution, CLRTAP/UNECE*), dentro del Programa de Cooperación Internacional de vegetación, cuyo objetivo es la evaluación y control de los efectos de la contaminación atmosférica sobre la vegetación (*Internacional Cooperative Programme on Effects of Air Pollution on Natural Vegetation and Crops*, *ICP-Vegetation*).

La financiación fue realizada por un convenio entre el Ministerio de Agricultura, Alimentación y Medio Ambiente (MAGRAMA) y el Centro de Investigaciones Energéticas Medioambientales y Tecnológicas (CIEMAT); y por distintos proyectos de investigación: BIOSTRESS (EVK2-C-1999-0004,EU), MONTES (CONSOLIDER-INGENIO CSD2008-00040, España), AGRISOST (S2009AGR-1630, Comunidad de Madrid, España), AGRISOST CM (P2013/ABI-2717, Comunidad de Madrid, España), y ECLAIRE (FP7-ENV, EU).

1. INTRODUCCIÓN

1.1. INTRODUCCIÓN GENERAL

Desde el comienzo de la revolución industrial, las emisiones de gases procedentes de las actividades antropogénicas se han ido incrementando, provocando efectos sobre los materiales, la vegetación, la salud humana y el medioambiente en su conjunto (Bergmann et al., 2015; Van Zelm et al., 2008). Si bien en un principio los problemas ocasionados tenían un componente local o regional, en la actualidad estas emisiones están alterando los complejos equilibrios físico-químicos que mantienen las propiedades y funciones de la atmósfera, afectando a la globalidad del sistema terrestre (Monks et al., 2009). Los cambios en la composición de la atmósfera pueden deberse bien a una modificación en la proporción de sus componentes naturales, bien a la introducción de nuevos compuestos.

Los contaminantes atmosféricos se clasifican en primarios y secundarios; los primarios, se emiten directamente desde sus fuentes, mientras que los secundarios son los que provienen de la transformación de los contaminantes primarios. En las últimas décadas, se ha intensificado el interés internacional por los contaminantes secundarios, dada la dificultad que entraña su control y por ser responsables de problemas de contaminación atmosférica que se extienden a escala regional o global. El ozono troposférico (O_3) es uno de los principales contaminantes atmosféricos pertenecientes a este grupo de contaminantes secundarios. Su incremento sobre el fondo natural se considera un componente clave dentro del conjunto de alteraciones ambientales derivadas de las actividades humanas sobre la Tierra, a las que se ha denominado en su conjunto como Cambio Global.

El O_3 es una forma alotrópica del oxígeno que se encuentra de forma natural en la atmósfera, tanto en la estratosfera, como en la troposfera. Su concentración es muy pequeña respecto a otros componentes naturales y es variable dependiendo de la altura. En la estratosfera alcanza su máximo de concentración, actuando como un filtro protector para la vida en la Tierra de la radiación solar ultra-violeta. En la troposfera, el O_3 se forma debido a un conjunto de complejas reacciones fotoquímicas entre contaminantes primarios, que se ven favorecidas bajo condiciones de estabilidad atmosférica y elevada radiación solar. Las emisiones antrópicas de precursores del O_3, tales como óxidos de nitrógeno (NO_x), compuestos orgánicos volátiles no metánicos

(COVNMs), monóxido de carbono (CO) y metano (CH_4), relacionados principalmente con fuentes industriales, agrícolas y transporte rodado, han incrementado significativamente el nivel de fondo natural del O_3 en superficie, especialmente en el hemisferio norte (Vingarzan, 2004). Bajo condiciones atmosféricas favorables, las concentraciones de este gas en la capa inferior de la atmosfera, alcanzan niveles que pueden tener consecuencias directas o indirectas en los materiales, la salud humana y la vegetación (CLRTAP, 2010). La gravedad de los efectos de este contaminante se debe a su gran capacidad oxidativa sobre las superficies con las que contacta (Notario et al., 2013). Además de esta toxicidad directa, este gas es responsable, junto con otros gases atmosféricos como el dióxido de carbono (CO_2), el metano (CH_4) y el óxido nitroso (N_2O), del calentamiento global del planeta por tratarse de un gas de efecto invernadero (Gauss et al., 2006).

Las primeras evidencias del carácter tóxico del O_3 en la vegetación se detectaron a finales de la década de los cincuenta en zonas agrarias de California (EEUU) afectadas de forma crónica por masas de aire contaminadas por oxidantes fotoquímicos, principalmente O_3. Se trataba de lesiones punteadas de tonos marrón-rojizo en el haz de las hojas de cultivos de viña (Richards et al., 1958), y de un moteado clorótico en hojas de tabaco (Heggestad and Middleton, 1959). Desde entonces, se han detectado daños foliares en muchas especies vegetales presentes en gran número de países (Mills et al., 2011), lo que verifica la toxicidad del contaminante a los niveles ambientales que se registran actualmente en algunas zonas del planeta. Actualmente el O_3 es considerado el contaminante atmosférico más fitotóxico, dada la gran cantidad de especies vegetales sensibles y la amplitud geográfica del problema (CLRTAP, 2010).

Los efectos de este contaminante en las plantas se pueden manifestar de distinta forma, como presencia de daños visibles en las hojas (Chaudhary & Agrawal, 2013), reducción del crecimiento de la biomasa aérea y radical (Hewitt et al., 2014, Vollness et al., 2010), aceleración de la senescencia y reducción del ciclo de vida (Dawnay & Mills, 2009), disminución de la capacidad reproductiva (Farre-Armengol et al., 2016) o pérdida de calidad nutritiva desde el punto de vista del aprovechamiento ganadero de los pastos (Gilliland et al., 2012). En los ecosistemas naturales, la gran heterogeneidad en la respuesta al O_3 entre especies de una misma comunidad, afecta a sus relaciones de competencia, alterando su estructura y composición y reduciendo su biodiversidad (Mills et al., 2011, Volk et al., 2016, Wedlich et al., 2012, Calvete et al., 2014, 2016).

Además, una exposición crónica al O_3 puede derivar en un debilitamiento de la vegetación, incrementando su susceptibilidad a otros factores bióticos o abióticos como la sequía, ataques de patógenos o déficit nutricionales (Weinstein et al., 1991; Foot et al., 1996; Grulke et al., 2008). A una escala mayor, también se ha determinado su efecto potencial sobre otros servicios ecosistémicos como la fijación de carbono (Witting et al., 2009; Ainsworth et al., 2012) o el balance de agua (McLaughlin et al., 2007).

Además del O_3, existen otras alteraciones en la atmósfera, derivadas de las actividades humanas, con capacidad para modificar los flujos de materia-energía de los ecosistemas y las relaciones de competencia entre los organismos, como es el depósito atmosférico de nitrógeno (N). Como consecuencia del incremento de las emisiones del transporte rodado, la quema de combustibles fósiles y la intensificación de la agricultura, se emiten a la atmosfera grandes cantidades de formas oxidadas de N (principalmente NO y NO_2) y reducidas (principalmente NH_3). El N es usualmente un nutriente limitante de la producción primaria en muchos ecosistemas, por lo que su incremento favorecería la producción vegetal. Sin embargo, el exceso de N puede sobrepasar la capacidad de asimilación del sistema y provocar una cascada de efectos ambientales, incluyendo la alteración de los procesos biogeoquímicos del suelo, la pérdida de biodiversidad y el aumento de la susceptibilidad a otros estreses bióticos o abióticos (Fenn et al., 2003; Stevens et al., 2004; Bobbink et al., 2010; Dupre et al., 2010; Sutton et al., 2011).

La cuenca mediterránea es una zona con elevadas emisiones de precursores de O_3, altos niveles de radiación solar, elevadas temperaturas y estabilidad atmosférica, que favorecen la producción fotoquímica de O_3. La dinámica atmosférica de los vientos en la cuenca favorece el transporte de masas de aire contaminadas, provocando concentraciones tóxicas de oxidantes fotoquímicos en zonas rurales y ecosistemas alejados de núcleos urbanos o fuentes contaminantes (Millán et al., 2002). En las últimas décadas, en los países del área mediterránea, se ha confirmado la fitotoxicidad del O_3 a concentraciones ambientales, como lo demuestra la presencia de síntomas visibles en distintos tipos de vegetación natural y agrosistemas (Mills et al., 2011). Además de los niveles elevados de O_3, la cuenca mediterránea cuenta con otro problema potencialmente grave derivado del depósito atmosférico de N. Este área se encuentra entre las más susceptibles al depósito de N, considerándose como una de las principales fuerzas de cambio en los ecosistemas mediterráneos que amenaza la biodiversidad de sus ecosistemas (Sala et al., 2000).

La conjunción de elevados niveles de O_3 en superficie y depósito de N atmosférico, es una de las situaciones más frecuentes bajo la que se encuentran los ecosistemas y agrosistemas mediterráneos. Sin embargo, el análisis experimental de los efectos que causa la mezcla de estos dos contaminantes es todavía muy escaso; aunque este conocimiento es clave para modelizar realísticamente la evolución de los ecosistemas en los escenarios futuros de cambio global y para la aplicación de políticas de control adecuadas.

La cuenca mediterránea se caracteriza por una gran heterogeneidad física: geológica, edáfica, climática y topográfica; esta variedad, unida a los distintos usos del territorio, producto de su compleja historia, han convertido a esta cuenca en una zona geográfica con niveles muy elevados de diversidad biológica y paisajística. Su riqueza de especies es tan elevada que se la incluye dentro de los 25 “puntos calientes” (*hotspots*) de biodiversidad a escala mundial (Myers et al., 2000). Uno de los ecosistemas mediterráneos más destacados es la dehesa, un sistema agroforestal formado por un dosel arbóreo disperso, bajo el cual se desarrollan comunidades pascícolas de las que se alimenta la fauna y la ganadería. Este sistema presenta una diversidad biológica mayor que otros tipos de hábitats naturales o manejados por el hombre, llegando a presentar del orden de 120 spp. 0.1 ha^{-1} (135 spp. ha^{-1} según Marañón, 1985; 146 spp. 0.1 ha^{-1} según García del Barrio et al., 2014). Su sistema de gestión es un ejemplo de conservación de la naturaleza, y a su vez, de una adecuada explotación de los recursos naturales, razones por las que este sistema está protegido bajo la Directiva Hábitat 92/43/EEC e incluida en la red Natura 2000. En la actualidad, en amplias zonas rurales de la Península Ibérica donde la dehesa forma parte de su paisaje, es frecuente la presencia de niveles de O_3 potencialmente tóxicos para la vegetación (Notario et al., 2014; Fernández-Fernández, 2014), y de un depósito de N en un rango de 10-38 kg ha^{-1} año (Sanz et al., 2002; Rodá et al., 2002; García-Gómez et al., 2014), un rango también por encima de los límites para asegurar la salud de este tipo de vegetación (Directiva de calidad de aire 2008/50/CE; CLRTAP, 2010; Bobbing et al., 2010).

La gravedad de los efectos tóxicos de los contaminantes atmosféricos, y del O_3 en particular, junto con su amplia distribución regional, llevó a la necesidad de establecer un programa a escala internacional para desarrollar políticas y estrategias de gestión medioambiental para el control de la contaminación atmosférica. Esta inquietud se materializó en 1979 en el Convenio de Naciones Unidas sobre Contaminación Atmosférica Transfronteriza a Gran Distancia (*Convention on Long-Range Transboundary Air Pollution*, CLRTAP/UNECE). Este convenio está firmado actualmente por 51 países, entre ellos España, y la UE con entidad propia. Este programa adoptó la metodología de niveles/cargas críticas para establecer la sensibilidad de los receptores a los contaminantes y establecer estrategias de reducción de la contaminación que eviten la superación de estos valores límites. Los niveles críticos (NCs) de O_3 (CL_e, *critical level* en sus siglas en inglés) para la protección de la vegetación, se definen como la exposición acumulada de O_3 (CL_{ec}) o el flujo estomático absorbido de O_3 (CL_{ef}) por encima del cual se producen efectos adversos sobre la vegetación sensible de acuerdo al conocimiento actual (CLRTAP, 2010). Los primeros NCs se establecieron en 1988, y son revisados periódicamente para incorporar los conocimientos científicos adquiridos para los distintos tipos de vegetación europea en base a los proyectos de investigación que se desarrollan de forma continua (CLRTAP, 2010). Estos NCs definidos en el Convenio se emplean para realizar análisis de riesgo de O_3 a escala europea y evaluar las políticas ambientales de calidad de aire; además, son la base de los valores objetivo establecidos en la directiva Europea de Calidad del Aire (2008/50/CE) para la protección de la vegetación.

En la presente memoria se estudia el efecto del O_3 en especies herbáceas anuales de comunidades pascícolas de dehesa, siguiendo la metodología del CLRTAP. En el primer capítulo de introducción, se describen los distintos aspectos del problema y su metodología de estudio. En el capítulo 2 se definen los objetivos, para cuyo desarrollo fueron necesarias tres fases experimentales y un análisis conjunto de toda la información. En el capítulo 3 se presentan los resultados mediante distintas publicaciones (SCI). En la discusión general, capítulo 4, se analiza y resume toda la información obtenida para cada una de las fases experimentales. Para finalizar en el capítulo 5 se expone de forma concisa las conclusiones de todo el trabajo. Es importante destacar el valor del análisis realizado en el capítulo 3.4 considerando toda la información, que permite cuantificar de forma global la respuesta al O_3 de este tipo de vegetación y la posibilidad de proponer niveles críticos de este contaminante para la protección de los pastizales anuales siguiendo la metodología del CLRTAP.

Todo el trabajo presentado está encuadrado en la línea de investigación que desarrolla el Grupo de Ecotoxicología de la Contaminación Atmosférica del Departamento de Medio Ambiente del CIEMAT, para el análisis de efectos de los contaminantes atmosféricos en comunidades de pastos mediterráneos. Los NCs propuestos en el último capítulo serán presentados en la próxima reunión anual del Grupo de Trabajo sobre Vegetación (*Internacional Cooperative Programme on Effects of Air Pollution on Natural Vegetation and Crops, ICP-Vegetation*) del CLRTAP para su aprobación e inclusión en los manuales técnicos del Convenio, con la finalidad de ser empleados en los análisis de riesgo para este tipo de vegetación en el ámbito mediterráneo.

1.2. LA CONTAMINACIÓN ATMOSFÉRICA

Existen muchas definiciones de contaminación atmosférica. La Agencia Europea de Medio Ambiente (EEA) la define como “la presencia en el aire de contaminantes o sustancias contaminantes en una concentración tal que interfiere con la salud o el bienestar humano, o que produce otros efectos medioambientales perjudiciales”. En la legislación española (Ley 34/2007), se define como: “La presencia en la atmósfera de materias, sustancias o formas de energía que impliquen molestia grave, riesgo o daño para la seguridad o la salud de las personas, el medio ambiente y demás bienes de cualquier naturaleza”. La alteración de la composición del aire puede tener un origen natural, producido por fuegos, erupciones volcánicas etc., o antropogénico, fundamentalmente debido a las emisiones derivadas de procesos industriales, agrícolas, urbanos y del transporte. El término de contaminación atmosférica se emplea de forma más habitual para referirse a los cambios en la composición química de la atmosfera causadas por la actividad humana.

Los contaminantes una vez emitidos a la atmosfera pueden sufrir transformaciones que alteran su naturaleza, por ello se clasifican en primarios, aquellos que se emiten directamente a la atmósfera y secundarios, aquellas especies que no se emiten directamente, sino que se forman a través de procesos químicos atmosféricos cuando los contaminantes primarios interaccionan entre sí y con los componentes naturales de la atmósfera. Los contaminantes más estudiados dentro de los primarios son: el monóxido de carbono (CO), los óxidos de nitrógeno (NO_2 y NO), los óxidos de azufre (SO_x, especialmente el SO_2), los compuestos orgánicos volátiles (COVs), que suelen dividirse en metano (CH_4) y compuesto orgánicos no metánicos (COVNMs), las partículas, el amoniaco (NH_3), el sulfuro de hidrógeno (H_2S), los halógenos (X_2), el dióxido de carbono (CO_2), el óxido nitroso (N_2O) y metales como el plomo (Pb) y el mercurio (Hg). A partir de estos contaminantes primarios se producen los principales contaminantes secundarios: el ozono (O_3), otros oxidantes fotoquímicos (cómo el PAN, Peroxiacetil Nitrato), sulfatos ($SO_4^{=}$) y nitratos (NO_3^-).

1.3. EL OZONO TROPOSFÉRICO

En 1840 el químico alemán C.F. Schönbein fue el primero en reconocer que el fuerte olor que se producía en algunos experimentos en los que se utilizaba oxígeno (O_2), se debía a la formación de una sustancia distinta derivada del O_2, a la que llamó ozono (del griego, oler). Posteriormente, en 1856, Th. Andrews demostró que esta sustancia estaba formada sólo por átomos de oxígeno, y finalmente en 1860 J.L. Soret estableció la relación entre el O_2 y el O_3, al determinar que tres volúmenes de O_2 producían dos de este gas. Por lo tanto, el O_3 es una forma triatómica del oxígeno molecular, con unas propiedades físicas y químicas que le confieren un fuerte carácter oxidativo, haciendo que sea una molécula fundamental en los procesos de la química atmosférica.

1.3.1. Distribución e importancia del ozono en la atmósfera

El O_3 es un componente natural de la atmósfera, cuya concentración depende de la altitud. En la estratosfera, se localiza el 90 % del total del O_3 atmosférico, donde puede alcanzar concentraciones de hasta 10 ppm. El O_3 que se encuentra en esta capa exterior actúa de filtro protector, evitando que la radiación ultravioleta alcance la superficie terrestre con una intensidad que pueda resultar nociva para la vida. Aunque otros compuestos como el O_2 filtran la radiación ultravioleta de longitudes de onda menor de 230 nm., solo el O_3 es capaz de atenuar las radiaciones por debajo de 290 nm. La emisión de compuestos clorofluorocarbonados (CFCs) de origen antropogénico, que reaccionan con el O_3 estratosférico y lo destruye, dio origen al fenómeno conocido como “agujero de la capa de O_3” sobre las zonas polares, siendo más conocido el problema en la Antártida. Las graves consecuencias que pudieron derivarse de este proceso, condujeron a la comunidad internacional a establecer restricciones en el uso de estas sustancias mediante la firma, en 1987, del Protocolo de Montreal (Chipperfield et al., 2015). Las observaciones actuales realizadas a nivel mundial han verificado que las concentraciones atmosféricas de las principales sustancias que destruyen la capa de O_3 han disminuido, y que la capa de O_3 mundial podría recuperar sus niveles de concentración naturales a mediados del presente siglo.

El otro 10 % de O_3 restante se distribuye en la troposfera. A nivel de superficie, los niveles de O_3 son mayores en las áreas urbanas, y sobre todo rurales, de las regiones más industrializadas, comparado con los que se registran en las regiones con menor desarrollo. Esto se debe a la concentración de actividades industriales, agrarias, de generación de energía y de transporte que emiten las sustancias precursoras del O_3 (Vingarzan, 2004). Cuando los niveles del O_3 en la superficie terrestre se elevan por encima del fondo natural, constituye un problema de contaminación atmosférica, dado que la elevada capacidad oxidativa de este contaminante puede resultar perjudicial para los materiales, la salud humana y los ecosistemas sobre los que se deposita (CLRTAP, 2010).

El O_3 es una sustancia fundamental en la dinámica de la química atmosférica, ya que participa en la producción de radicales hidroxilo OH^* y HO_2^* que son especies altamente oxidantes y que inician las cadenas de oxidación en la mayoría de los gases reducidos. Sin el O_3, gases reducidos como el CO, los hidrocarburos y la mayoría de los compuestos de azufre y nitrógeno reactivos, se acumularían considerablemente en la atmósfera.

El incremento de la concentración de O_3 también tiene repercusión en el calentamiento global del planeta, ya que tiene propiedades de gas de efecto invernadero como el dióxido de carbono (CO_2), el metano (CH_3) y el óxido nitroso (N_2O) (Gauss et al., 2006). El O_3 se encuentra en la tercera posición entre los gases con mayor capacidad para alterar el flujo radiativo de la atmosfera (forzamiento radiativo).

1.3.2. Procesos atmosféricos de formación de ozono

La reacción química más significativa que produce O_3 en la atmósfera es la que transcurre entre el oxígeno atómico y el molecular.

En la estratosfera, por encima de los 20 kilómetros de la superficie terrestre, los átomos de oxígeno provienen de la fotodisociación del oxígeno molecular por la acción de la radiación en el ultravioleta lejano:

$O_2 + hv \rightarrow 2O$ ($\lambda < 240$ nm)

El oxígeno atómico se combina con el molecular produciendo O_3 según la reacción:

$O_2 + O + M \rightarrow O_3 + M$

Esta reacción es exotérmica y no requiere la absorción de un fotón. La molécula M, puede ser entre otras N_2 u O_2 que eliminan la energía de la reacción y estabilizan la molécula de O_3. Este O_3 formado puede destruirse fotoquímicamente, ya que absorbe en la región media del ultravioleta produciendo una inversión de la reacción anterior. El oxígeno atómico formado puede posteriormente combinarse con el O_3 para producir oxigeno molecular, iniciándose el ciclo.

$O_3 \rightarrow O_2 + O$ ($200 < \lambda < 300$ nm)

$O + O_3 \rightarrow 2O_2$

En la troposfera, la única producción significativa de oxígeno atómico proviene de la fotodisociación del NO_2 por la radiación solar en el ultravioleta cercano (Chameides & Walker, 1973; Crutzen, 1974):

$NO_2 + hv \rightarrow NO + O$ ($\lambda < 430$ nm)

El oxígeno resultante reacciona con el molecular formando O_3:

$O_2 + O + M \rightarrow O_3 + M$

En ausencia de otras reacciones competitivas, la rápida reacción del NO con el O_3 completa el ciclo regenerando una molécula de NO_2 según la reacción:

$NO + O_3 \rightarrow NO_2 + O_2$

En ausencia de otros oxidantes, el O_3 oxidará al NO para regenerar el NO_2 sin ganancia neta de O_3, por lo que sólo existirá una acumulación de O_3 si existe un paso alternativo que evite su consumo. Esa alternativa viene dada por diversas reacciones fotoquímicas que tienen lugar por la presencia de otros compuestos atmosféricos y dan lugar a la formación neta de O_3 y otros oxidantes fotoquímicos. Existen tres mecanismos básicos para que esto ocurra: la oxidación de los hidrocarburos no metánicos (HCNMs) dentro de los COVs, el monóxido de carbono (CO) y el metano (CH_4). Los mecanismos de reacción se inician con el oxidante primario hidroxilo (OH*). En todos los casos, los mecanismos son catalíticos con respecto a los NO_x, ya que se produce inter-conversión de NO a NO_2 sin pérdida de NO_x. Sin embargo, el CO, el CH_4 y los COVs se consumen en las reacciones y por lo tanto son considerados el combustible de la reacción.

La producción de O_3 a través de los COVs se produce por su oxidación mediante la reacción con radicales hidroxilo que producen radicales peróxilo orgánicos (RO_2*):

$RH+OH^*\rightarrow H_2O+R^*$

$O_2+R^*\rightarrow RO_2^*$

Los radicales peróxilo presentan una mayor afinidad de reacción con el NO que el O_3; por ello en su presencia son los que oxidan el NO en lugar del O_3. De este modo, la presencia de COVs produce la acumulación de O_3 en las capas bajas de la atmósfera, ya que el O_3 no se consume al ser éste desplazado por los radicales RO_2* durante el proceso de oxidación del NO (Chameides & Walker, 1973; Crutzen, 1973).

1.3.3. Procesos atmosféricos de destrucción de ozono

Los principales procesos químicos atmosféricos de eliminación del O_3 troposférico son fundamentalmente las reacciones químicas con oxidantes y la fotólisis.

Cuando las concentraciones de NO_x son cercanas a cero, los mecanismos expuestos anteriormente no forman O_3 y las reacciones de los radicales compiten con las siguientes reacciones que interconvierten las especies HO_x y conducen a una pérdida catalítica de O_3 (Crutzen, 1973):

$HO_2^* + O_3 \rightarrow OH^* + 2O_2$

$OH^* + O_3 \rightarrow HO_2^* + O_2$

En ausencia de NO_x, la oxidación del CO puede también llevar a un proceso de destrucción de O_3 a través de las siguientes reacciones encadenadas:

$OH^* + CO \rightarrow CO_2 + H^*$

$H^* + O_2 + M \rightarrow HO_2^* + M$

$HO_2^* + O_3 \rightarrow OH^* + 2O_2$

Si el HO_2^* reacciona con otro radical peroxilo en lugar de reaccionar con el O_3, no se produciría un cambio en la concentración de O_3.

El proceso de fotólisis predomina en las regiones remotas de baja altitud, donde la concentración de vapor de agua en la atmósfera puede ser importante. El mecanismo de reacción que da como producto el radical hidroxilo, OH, es el que sigue:

$O_3 + hv\ (< 320\ nm) \rightarrow O_2 + O$

$O + M \rightarrow O + M$

$O + H_2O \rightarrow 2OH^*$

Muchos de los átomos de oxígeno electrónicamente excitados se convierten a átomos de oxígeno no excitados que se transforman rápidamente en O_3, pero unos pocos de los átomos excitados reaccionan con el vapor de agua para producir radicales hidroxilo (Guicherit & Roemer, 2000).

1.3.4. Fuentes y sumideros del ozono troposférico

Además de los procesos de producción fotoquímica de O_3 y de la destrucción química del mismo comentado en los apartados anteriores, el O_3 troposférico tiene otras fuentes y sumideros. Entre las fuentes naturales generadoras se encuentran la producción de O_3 en la estratosfera y su inyección a la troposfera por los movimientos de circulación del aire entre capas. Las entradas de O_3 desde la estratosfera suelen caracterizarse por incrementos bruscos en su concentración, aunque pueden no detectarse fácilmente en superficie dado que los procesos de mezcla de la troposfera diluyen el O_3 inyectado al distribuirlo de forma relativamente uniforme. Se estima que más de la mitad del O_3 inyectado sería mezclado y diluido por encima de los 3 km de altura. A pesar de esto, los niveles elevados de primavera que se registran en zonas rurales se han asociado frecuentemente con este tipo de intrusiones estratosféricas (Cristofanelli et al., 2015).

Los procesos físicos que eliminan O_3 en superficie se dividen entre depósito seco, por el que el O_3 reacciona y se destruye irreversiblemente en el suelo, el agua o la superficie de la vegetación; y el depósito húmedo, por el que el contaminante se incorpora a elementos de precipitación como nubes, gotas de lluvia y aerosoles, aunque este último es de poca importancia debido a la baja solubilidad del O_3 en agua.

El depósito seco, cuantitativamente el más importante, resulta de la reacción del O_3 con las superficies del suelo y la vegetación, y en gran medida de su absorción a través de los estomas de las plantas. Por ello, aunque las tasas de depósito seco de un gas atmosférico dependen de las propiedades de la superficie y de las características propias del gas, son muy importantes las variables meteorológicas de las que depende la apertura estomática que determina el flujo de O_3 al interior vegetal, como la temperatura, el déficit de presión de vapor (*vapour pressure déficit*, VPD), la disponibilidad de agua en el suelo y la radiación solar (González-Fernández et al., 2010). La estructura foliar, la edad y el estado fenológico de la vegetación son también factores importantes a considerar, junto con la velocidad del viento, la turbulencia o la presencia de agua sobre las superficies; todos ellos determinantes en la cuantificación del depósito seco O_3 sobre el dosel vegetal (González Fernández et al., 2010). Además, el depósito de O_3 sobre la superficie vegetal puede estar influido por otros gases emitidos por la propia vegetación como son los COVs que reaccionan con el O_3 (Fares et al., 2010).

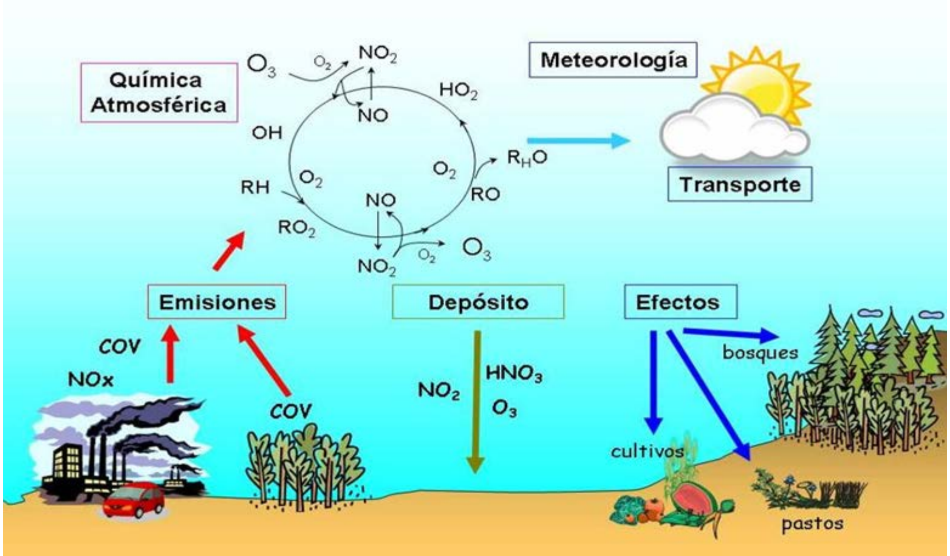

Figura 1. Ciclo sintetizado de formación del ozono y procesos atmosféricos relacionados. Fuente: El ozono troposférico y sus efectos en la vegetación. Ministerio de Medio Ambiente y Medio Rural y Marino. Madrid, 2009.

1.3.5. Variabilidad temporal y espacial del ozono troposférico

La concentración de O_3 en la atmósfera es el resultado del equilibrio dinámico de los procesos de formación, transporte, depósito y destrucción, que son dependientes de un conjunto de factores como la emisión de los compuestos precursores, la distancia de los focos de emisión, la topografía, la cubierta vegetal y las condiciones meteorológicas (Millán et al., 2002).

Los niveles de O_3 reproducen comportamientos característicos diarios y estacionales, además de fluctuaciones interanuales. En el ciclo diario característico bajo influencia de emisiones procedentes de áreas industriales y urbanas, los niveles de O_3 comienzan a incrementarse desde primeras horas de la mañana, con el comienzo de la emisión de sus precursores, alcanzándose los máximos pasado el mediodía, coincidiendo con las horas de máxima insolación y temperatura. Tras alcanzarse estos máximos, los niveles comienzan a decrecer de forma progresiva a lo largo de la tarde, manteniendo esta pauta decreciente durante la noche debido a la disminución de la temperatura y radiación, y a la intensificación de los procesos de destrucción de O_3 (Chameides & Walker, 1973; Crutzen, 1973). En otras localizaciones en los que la influencia de las fuentes directas de emisión es prácticamente nula, los niveles de O_3 permanecen prácticamente constantes durante todo el día, incrementándose levemente a media tarde. Este tipo de ciclo es frecuente en zonas de montaña de media-gran altitud:

ciclos muy amortiguados con pequeñas oscilaciones diarias, especialmente durante los meses más fríos (Elvira et al., 2016).

La variación estacional de la concentración de O_3 en zonas afectadas por fuentes precursoras presenta los máximos en primavera-verano, cuando las condiciones climatológicas, mayor temperatura, radiación solar y estabilidad atmosférica, son más favorables para la producción fotoquímica del contaminante. En zonas remotas, sin focos precursores cercanos, el ciclo anual presenta a finales de invierno y principios de primavera sus máximos, manteniéndose elevados durante el verano (Elvira et al., 2016).

Las variaciones anuales de las condiciones meteorológicas, así como de las emisiones de los precursores, pueden provocar fluctuaciones interanuales en las concentraciones medias, máximas y mínimas, dificultando la definición de patrones generalizables y las políticas de control de la contaminación atmosférica.

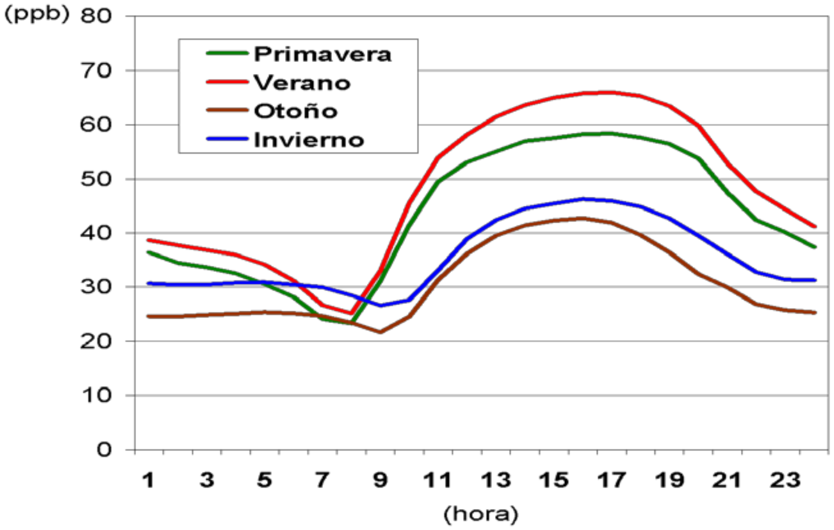

Figura 2. Perfil diario medio de O_3 para las distintas estaciones del año durante el período 2004-2006 en la localidad de Buitrago de Lozoya (975 metros, Madrid). Datos procedentes de la Red de Calidad del Aire de la Comunidad de Madrid.

Los primeros estudios relacionados con la contaminación fotoquímica por O_3 tuvieron como objetivo el análisis de su comportamiento en entornos urbanos e industriales, principales focos emisores de precursores, y donde en teoría debían alcanzarse las concentraciones más elevadas. Sin embargo, estudios posteriores en zonas rurales o alejadas de focos de emisión, demostraron que los niveles de O_3 generalmente superaban los valores registrados en las zonas de emisión. Esto es debido a la importancia del transporte de O_3 regional e incluso continental, y al envejecimiento que sufren las masas de aire contaminadas durante su transporte, que conlleva en condiciones meteorológicas favorables para la actividad fotoquímica, un importante enriquecimiento en O_3 (Millán et al., 2002). De este modo, el O_3 alcanza en superficie valores más elevados en zonas alejadas de los focos precursores, constituyendo un problema a escala regional y global.

1.3.6. Evolución del fondo de ozono troposférico

Las primeras medidas de O_3 en superficie se realizaron en emplazamientos de Europa y América a finales del siglo XIX. Las series de datos de estas estaciones son limitadas debido a que la mayoría no tuvieron continuidad y utilizaron el método Schoenbein de medida que presentaba serias carencias analíticas. A pesar de estas limitaciones, las correcciones y análisis posteriores de estos datos han servido para estimar los niveles de fondo de O_3. Estos primeros promedios anuales se encuentran próximos a 10 ppb. (Marenco et al., 1994; Anfossi et al., 1991; Sandroni et al., 1992), si bien es cierto que estos valores no debían de carecer completamente de la influencia de las emisiones antrópicas, han sido considerados como los valores de fondo natural de O_3.

Desde la revolución industrial, el incremento de las emisiones ha sido de tal magnitud que se han producido cambios en la composición de la atmósfera con repercusiones a escala global (Monks et al., 2009). Como consecuencia del aumento de las emisiones de forma mayoritaria en el hemisferio norte, se ha multiplicado por dos los niveles de fondo de O_3 en este hemisferio desde finales del siglo XIX: a latitudes medias europeas el promedio anual se encuentra actualmente en el rango de 20-45 ppb; en Canadá y Estados Unidos entre 23-34 ppb (Vingarzan, 2004). Se ha estimado que durante las últimas décadas, los niveles de fondo de O_3 han aumentado siguiendo una tasa de incremento anual en el rango 0.5-2 %; aunque de forma más reciente, se ha producido una progresiva ralentización coincidiendo con las restricciones impuestas en Europa sobre las emisiones de precursores de O_3 (Vingarzan, 2004; Guicherit el al., 2000).

El transporte intercontinental de O_3 y sus precursores también es un factor fundamental en la determinación de los niveles de fondo de este contaminante. En las latitudes medias del hemisferio norte, el fondo de O_3 está muy determinado por el transporte desde la zona asiática y por el incremento de las emisiones principalmente del sureste asiático, China e India (Oltmans et al., 2006).

Las predicciones futuras de las concentraciones de O_3 en superficie se encuentran ante el gran reto que supone modelizar el conjunto de cambios globales, como el cambio de uso del suelo, el calentamiento global o el cambio climático, que interaccionan y afectan a los procesos de formación, eliminación y transporte del O_3 y sus precursores (Cape, 2008). Stich et al., (2007) en sus predicciones para el año 2100 estiman una concentración media alrededor de los 75 ppb. en gran parte de Europa, y de 90 ppb. en áreas de Suramérica, África y el sureste asiático, lo que implicaría un importante agravamiento a escala global del problema ambiental que constituye el incremento de los niveles de O_3 troposférico.

1.3.7. Niveles de ozono troposférico en la Península Ibérica

Las condiciones climáticas del sur de Europa facilitan los procesos fotoquímicos atmosféricos de producción de O_3. Por ello, los niveles que se registran en los países de la cuenca mediterránea se encuentran entre los más altos de Europa, como queda reflejado en el mapa de la Agencia Europea de Medio Ambiente (figura 3).

En la península Ibérica, en las dos últimas décadas se han realizado numerosos trabajos con la finalidad de caracterizar la dinámica y los niveles de O_3 en superficie en diversas áreas rurales y urbanas. Los primeros estudios se efectuaron en la Comunidad de Madrid (Plaza et al., 1997) y en la costa mediterránea, donde las emisiones de precursores, los factores geográficos y meteorológicos favorecen la producción de O_3 (Millán et al., 1997, Millán et al., 2002; Ribas & Peñuelas 2003; Dueñas et al., 2004). Posteriormente se han ido ampliando los estudios de niveles de O_3 troposférico hacia áreas del sur-oeste (Adame et al., 2008, Adame et al., 2010) y otras áreas del interior peninsular (García et al., 2005, Notario et al., 2012; Notario et al., 2013; Villanueva et al., 2014; Elvira et al., 2016). En la costa mediterránea se observaron los primeros síntomas de O_3 en vegetación natural y cultivos (Gimeno et al., 1999).

Los trabajos más recientes indican que en la actualidad se está excediendo en la mayor parte de la península, y de forma crónica, los valores límite de O_3 para asegurar la protección de la vegetación de acuerdo a la Directiva de Calidad de Aire Europea (2008/50/CE) (Fernández-Fernández et al., 2011). Además, es interesante considerar que la mayoría de los trabajos mencionados indican la presencia de valores elevados del contaminante durante los meses de primavera, coincidiendo con el periodo de máxima actividad fisiológica de la vegetación de carácter mediterráneo, incrementando su daño potencial.

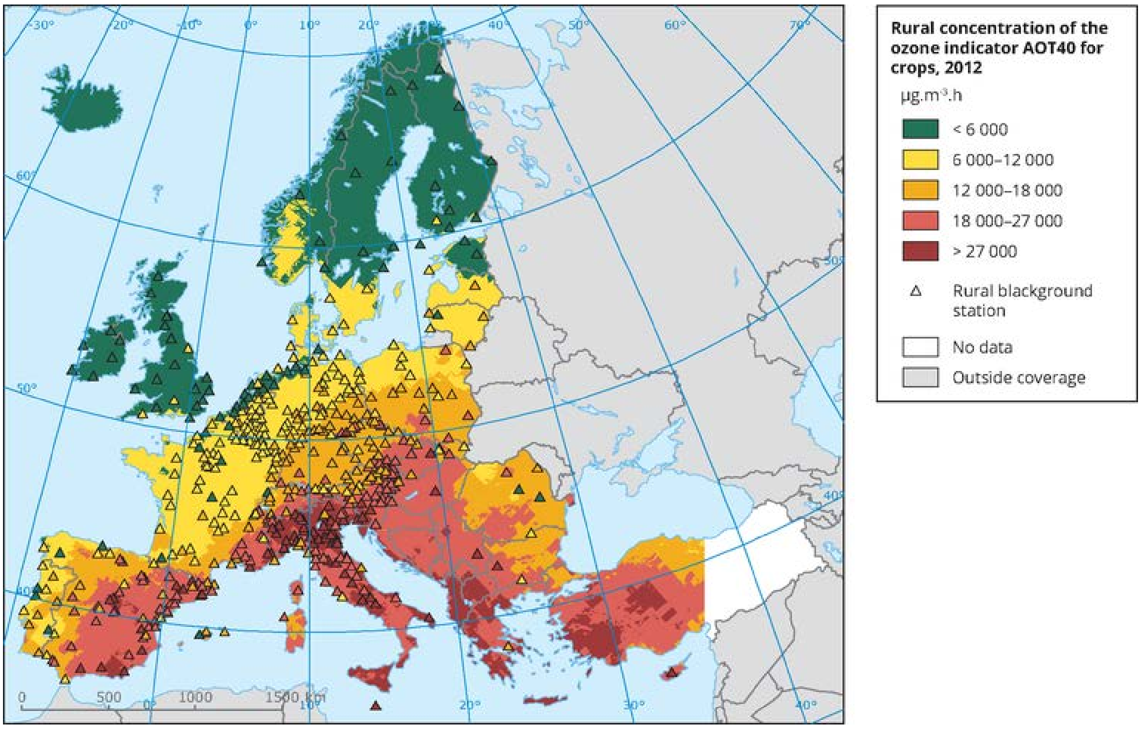

Figura 3. Concentración de O_3 en Europa expresada en base al índice acumulado durante 3 meses (mayo-julio) AOT40 durante el año 2012. Fuente: European Environment Agency.

1.4. EFECTOS DEL OZONO EN LA VEGETACIÓN

A finales de la década de los cincuenta, se detectaron en California (EEUU) las primeras evidencias del carácter tóxico del O_3 en la vegetación. Se describieron por primera vez síntomas foliares específicos inducidos por concentraciones elevadas de O_3, que consistían en lesiones punteadas de tono marrón-rojizo en el haz de hojas de cultivos de viña (Richards et al., 1958), y de un moteado clorótico en hojas de tabaco (Heggestad and Middleton, 1959). Desde entonces, se han detectado este tipo de daños foliares en muchas especies vegetales en un gran número de países (Mills et al., 2011), verificando la toxicidad del contaminante a los niveles que se registran actualmente en algunas zonas del planeta. En la actualidad, se considera al O_3 como el contaminante atmosférico más fitotóxico, dada la gran cantidad de especies vegetales sensibles y la amplitud geográfica del problema (CLRTAP, 2010).

Figura 4. Síntomas foliares característicos en trébol subterráneo (*Trifolium subterraneum*) inducidos por la exposición a O_3 durante los experimentos desarrollados en OTCs (Autor: M. Mendoza/CIEMAT).

1.4.1 Mecanismo de acción del ozono a escala celular. Sistemas de defensa

El principal mecanismo de acción del O_3 es mediante la formación de las especies reactivas de oxígeno (EROs) al entrar en contacto con el medio acuoso y reaccionar con las moléculas presentes en la estructura de la hoja. Las EROs son un término genérico que incluye diferentes especies de radicales libres de oxígeno molecular (O_2): el radical superóxido (O_2^-), el radical hidroxilo (OH^-), el oxígeno en singlete ($^1O_2$) y además al peróxido de hidrógeno (H_2O_2). Los radicales libres son especies químicas que contienen uno o más electrones no apareados en su última capa lo cual les permite reaccionar con un elevado número de moléculas de todo tipo, oxidándolas y con ello alterando sus estructuras (Halliwell, 2006). En las plantas, la formación de EROs se produce continuamente durante los procesos metabólicos habituales de la célula, como subproductos de diferentes rutas metabólicas con actividad oxidante alta o con una intensa tasa de flujo electrónico, y se localiza en distintos orgánulos, principalmente en cloroplastos, mitocondrias y peroxisomas (Del Rio et al., 2006). Esta es la razón por la cual las plantas disponen de mecanismos de defensa en forma de enzimas y moléculas frente a las EROs para evitar que se produzcan daños sobre sus componentes celulares (Navrot et al., 2007). Las principales enzimas vegetales que protegen frente a la actividad de los radicales oxidantes son la superóxido dismutasa, la catalasa, la peroxidasa y la ascorbato peroxidasa. Entre las moléculas antioxidantes destacan entre otros, el ácido ascórbico o vitamina C, el glutatión y los compuestos fenólicos. En condiciones normales, en las células de la planta existe un equilibrio entre la formación y la destrucción de EROs, pero se puede perturbar por una sobreproducción ("explosión oxidativa") de EROs provocado por distintos estreses de tipo abiótico o biótico como puede ser la: salinidad, radiación UV, sequía, metales pesados, contaminantes atmosféricos, temperaturas extremas, déficit de nutrientes, ataques de patógenos y herbicidas (Singh Gill & Tuteja, 2010). Los EROs tienen una función importante en el inicio de la respuesta hipersensible, una forma de muerte celular programada, que implica la inducción de expresión de genes de defensa que responden a las alteraciones que provocan los estreses anteriormente citados (Singh Gill & Tuteja, 2010).

El O_3, una vez en el interior de la cavidad sub-estomática, entra en contacto con moléculas presentes en el apoplasto, pared celular y membrana plasmática, desencadenando una sucesión de reacciones que producen EROs y otros compuestos tóxicos, capaces de reaccionar y oxidar multitud de compuestos orgánicos, provocando diversas alteraciones estructurales, que conducen a otros tipos de alteraciones funcionales, afectando a la fisiología de las células y en consecuencia a la planta en general, efecto conocido como estrés oxidativo (Laisk *et al.*, 1989; Pryor & Church, 1991). El estrés oxidativo que provoca el O_3 a escala celular induce una respuesta de la planta que se asemeja a la que producen otros factores de estrés como los mencionados anteriormente. La primera línea de defensa contra el O_3 se encuentra en la capacidad antioxidante del apoplasto, especialmente basada en la concentración de ascorbato y de su capacidad de regeneración, aunque el resto de compuestos antioxidantes comentados anteriormente también tienen un papel muy importante en los procesos de detoxificación y tolerancia al contaminante (Dizengremel et al., 2008).

A escala celular, las primeras moléculas en reaccionar con el O_3 y los radicales libres generados, son los lípidos de la membrana plasmática, aminoácidos de las proteínas de las membranas y una gran variedad de metabolitos localizados en la pared celular (Fiscus et al., 2005). El primer resultado es una disfunción de las propiedades de la membrana plasmática, que unido a otras reacciones en cadena provocan cambios en el metabolismo celular. Entre los efectos causados sobre este metabolismo se han descrito cambios en la concentración de iones como el calcio, pérdida de agua, cambios en la concentración y la actividad de enzimas, estimulación de la producción de compuestos antioxidantes y de etileno, muerte celular, etc. (Pell *et al.*, 1997; Schraudner *et al.*, 1997; Skärby *et al.*, 1998; Black *et al.*, 2000; Fiscus *et al.*, 2005; Heath, 2008). El O_3 provoca cambios en la expresión de diversos genes (Pell *et al.*, 1997; Heath, 2008). La señal percibida por la célula ante el estrés oxidativo que produce el O_3, induce cambios que regulan la transcripción de estos genes. Sin embargo las rutas no son únicas ni específicas para el contaminante, ya que diferentes estreses pueden provocar los mismos mecanismos de respuesta a escala de expresión génica (Schraudner *et al.*, 1997; Heath, 2008).

Los estudios recientes han demostrado la importancia de las emisiones de isoprenoides, especialmente de monoterpenos y sesquiterpenos, dos compuestos orgánicos volátiles que reaccionan rápidamente con el O_3, como mecanismo clave en la protección de la planta (Bouvier-Brown et al., 2009).

Las plantas expuestas a niveles elevados de O_3 incrementan su producción de moléculas y enzimas antioxidantes para reducir el medio oxidante (Kangasjarvi et al., 1994). Sin embargo, estos sistemas están estrechamente ligados a las condiciones ambientales y el estado fisiológico de la planta, por lo que la actividad de los mecanismos de defensa y detoxificación presenta importantes variaciones diarias y estacionales e interacciones con otros estreses ambientales (Elvira et al., 1998; Alonso *et al.*, 1999; Alonso *et al.*, 2001). Estas variaciones se relacionan con la variabilidad de la respuesta de la vegetación al O_3, que puede cambiar en función de las condiciones ambientales, la fenología y el estado fisiológico de la vegetación.

Los mecanismos de defensa celular que están relacionados con la tolerancia al O_3 presentan una gran complejidad y especificidad que, en la actualidad y en base a la información disponible, no permite generalizar un patrón de respuesta para incluirlo dentro de los modelos tipo DO_3SE (CLRTAP/UNECE), que estiman la respuesta de la vegetación al O_3 (explicado con más detalle en el apartado 1.5.5).

1.4.2. Efectos del ozono en la fisiología de la vegetación

Existe una gran variabilidad en la tolerancia de las plantas al O_3 entre familias, géneros, especies, poblaciones y variedades. Estas diferencias se deben a sus distintas estrategias de defensa frente al contaminante, evitando la absorción del O_3 o reparando el daño que produce. En general, las especies más tolerantes presentan parámetros que limitan la absorción de O_3, como una conductancia estomática reducida, pero una vez generados los EROs, la capacidad potencial de detoxificación y reparación a escala celular es crucial para combatir al estrés oxidativo y favorecer el restablecimiento del equilibrio celular (Massman, 2004; Wieser&Matissek, 2007). Cuando no se consigue reestablecer este equilibrio, los EROS provocan diversas alteraciones estructurales, que conducen a otros tipos de alteraciones funcionales, afectando a los procesos metabólicos, fisiológicos y finalmente al crecimiento y producción vegetal.

La plantas sensibles expuestas al contaminante pueden desarrollar daños foliares específicos, que en una exposición aguda pueden aparecer en pocas horas o días, mientras que en exposiciones crónicas, que son las más habituales, se desarrollan más lentamente a lo largo de semanas desde el inicio a la exposición. Sin embargo, puede ocurrir que la planta esté sometida a un estrés oxidativo, afectando a su funcionamiento fisiológico, sin observarse síntomas foliares visibles.

Entre los principales procesos fisiológicos afectados por el O_3, se incluyen la actividad fotosintética, la translocación y el reparto de los asimilados (Andersen, 2003; Fuhrer y Booker, 2003; Ashmore, 2005; Fiscus *et al.*, 2005).

La reducción de la tasa de asimilación que se detecta como un efecto característico de la planta expuesta a niveles elevados de O_3, se ha relacionado con distintos mecanismos afectados por el contaminante, entre ellos: descenso en la actividad de la Rubisco, reducción en el contenido de clorofila, alteraciones de la cadena transportadora de electrones de la fotosíntesis, daños en las células guarda que controlan la apertura estomática, o un aumento de la tasa de respiración posiblemente relacionado con una intensificación de los mecanismos de protección y reparación (Darral, 1989; Barnes *et al.*, 1990; Peñuelas *et al.*, 1994; Schraudner *et al.*, 1997; Andersen, 2003; Fiscus *et al.*, 2005; Feng *et al.*, 2008; Heath, 2008).

Los patrones de translocación y distribución de los productos asimilados también pueden resultar afectados por el O_3. Entre los mecanismos que se han descrito para explicar los efectos sobre estos procesos se encuentran: la alteración de la carga del floema, el aumento de la demanda de asimilados por los sistemas de detoxificación y reparación, que se traducen en aumentos de la tasa de respiración, y los desequilibrios en el balance del reparto entre las fuentes y los sumideros de carbono dentro de la planta debido a la reducción de la tasa de asimilación y del aumento de la demanda de asimilados en las hojas (McLaughin y McConathy, 1983; Andersen, 2003).

Las implicaciones de los cambios en el patrón de reparto de asimilados pueden tener consecuencias importantes a largo plazo, al provocar alteraciones en el desarrollo del dosel o las raíces que traen consigo cambios en el acceso a los recursos (nutrientes, agua), afectando a la vitalidad de las plantas o las relaciones de competencia entre especies (Skärby *et al.*, 1998; Ashmore, 2005). Puesto que el reparto de los asimilados depende tanto de la actividad de los sumideros (tallos, raíces), como de las tasas de asimilación en hojas, los efectos del O_3 pueden depender de las prioridades del reparto de asimilados en el momento de la exposición al contaminante (Ashmore, 2005). En el caso del trigo, la antesis y la maduración del grano son los periodos más sensibles al O_3 por su repercusión negativa en la producción (Pleijel *et al.*, 1998). En cambio, en otras especies como el tomate, el periodo vegetativo es el más sensible. En este caso, la exposición temprana al O_3 provoca una reducción de la biomasa, que altera la tasa entre biomasa aérea y subterránea, y un retraso en el desarrollo fenológico, afectando a la producción temprana del cultivo (Bermejo, 2002). A veces, el descenso o la pérdida de la actividad de las hojas más viejas puede ser compensada mediante la producción de hojas nuevas, con mayores tasas de fotosíntesis, pero esto también se realiza a expensas del reparto de carbono hacia otros sumideros (Ashmore, 2005).

Entre las consecuencias de la reducción del flujo de productos asimilados hacia los sumideros se ha destacado el impacto sobre las raíces, por sus consecuencias en gran cantidad de procesos del sistema planta-suelo. Esta reducción del flujo de carbono hacia las raíces debida a la exposición al O_3, puede alterar su función hidráulica, lo que afecta a las relaciones hídricas de toda la planta y a la absorción de nutrientes del suelo. Además también disminuye la cantidad de exudados orgánicos liberados de las raíces al suelo, afecta a la fijación de nitrógeno de las leguminosas y al desarrollo de las micorrizas, y en general provoca una pérdida de vigor de las plantas e incrementa su sensibilidad frente a otros factores de estrés (Skärby *et al.*, 1998; Andersen, 2003; Fuhrer y Booker, 2003; Ashmore, 2005).

1.4.3. Modulación del efecto del ozono por la disponibilidad de nitrógeno

Hasta comienzos del siglo XX, las reacciones atmosféricas provocadas por los rayos, las emisiones volcánicas, y la fijación biológica de las leguminosas, eran las principales vías de entrada de N a los ecosistemas, lo que le convertía en uno de los principales limitantes de la productividad vegetal. El uso de combustibles fósiles en actividades industriales y transporte, así como la intensificación de los sistemas agrarios, y uso de fertilizantes nitrogenados sintéticos procesados de forma industrial mediante el método de Haber-Bosch, han sido responsables de la emisión de grandes cantidades de formas oxidadas y reducidas de N a la atmósfera, convirtiendo cantidades inmensas de nitrógeno atmosférico estable (N_2) en formas reactivas que pueden llegar a convertirse en formas asimilables para la vegetación (Gruber & Galloway, 2008).

1.4.3.1. Depósito de nitrógeno atmosférico

Los principales compuestos de N emitidos a la atmósfera por actividades antrópicas son el amoníaco (NH_3), principalmente por la agricultura y ganadería, y los óxidos de nitrógeno (NO_x), que incluyen NO y NO_2, emitidos por el transporte, la generación de energía eléctrica y otras fuentes de combustión. Las emisiones de óxido nitroso (N_2O), gas de efecto invernadero, son menores y provienen principalmente del suelo agrícola (Snyder et al., 2009). Una vez emitidos a la atmosfera, estos compuestos nitrogenados se transportan y depositan en forma seca o húmeda en los ecosistemas terrestres y acuáticos. La cantidad de su depósito depende de una combinación entre las características de las superficies receptoras, las condiciones ambientales y la naturaleza química de la molécula que se deposita. Como consecuencia de este depósito de origen antrópico, se ha visto afectado de forma importante el ciclo del N, causando una cascada de problemas medioambientales que incluyen la acidificación de los suelos y la eutrofización de los ecosistemas (ENA, 2011).

Inicialmente el incremento de N en los ecosistemas provoca un incremento de la biomasa vegetal, que podría ser interpretado como positivo, pero a largo plazo puede llegar a provocar cambios estructurales en las comunidades vegetales provocando pérdida de biodiversidad, uno de los riesgos más importantes asociados al incremento del depósito de N (Bobbink et al., 2010). Se ha caracterizado un ejemplo muy claro de este fenómeno en las praderas ácidas del norte de Europa (Stevens et al., 2004 and 2006; Dupre et al., 2010). En el área mediterránea, los estudios realizados con herbáceas no parecen relacionar directamente el incremento del depósito de N con la pérdida de biodiversidad. Por el contrario, Dias et al (2011) observaron un incremento de la diversidad de plantas vasculares, provocado por el aumento en la disponibilidad de N en un suelo extremadamente pobre en nutrientes; mientras que Bonamoni et al (2009), en un pastizal desarrollado en suelo calizo no detectaron cambios en la diversidad con el enriquecimiento de N en suelo.

1.4.3.2. Interacción entre ozono y depósito de nitrógeno atmosférico

En la actualidad es frecuente que los ecosistemas estén bajo el efecto de distintos contaminantes atmosféricos. La combinación de niveles elevados de O_3 y depósito de N es una de las combinaciones más frecuentes con capacidad para alterar el equilibrio de los ecosistemas (Takemoto et al., 2001). La respuesta de la planta a esta combinación no es fácil de predecir ya que, de forma individual, las plantas presentan respuestas contrarias a ambos contaminantes: el N estimula el crecimiento, mientras que el O_3 lo disminuye. Uno de los estudios más completos sobre este tema se realizó en las montañas de San Bernandino (California). En esta zona, se ha caracterizado la respuesta de un bosque mixto de coníferas a los niveles altos de O_3 y depósito de N. Entre los efectos observados se encuentran una disminución de la biomasa de raíces finas, desequilibrios en la concentración de C y N foliar y alteración del balance de agua; efectos que favorecieron la susceptibilidad del bosque a otros estreses bióticos y abióticos y, todos ellos combinados, contribuyeron a una mayor sensibilidad del ecosistema al fuego (Grulke et al., 2009, 1998; Jones et al., 2004).

En un experimento de tres años que evaluó los efectos del O_3 y el N en una comunidad natural de pastos subalpinos, no se detectaron efectos significativos en productividad y composición florística de la comunidad debidos al incremento de O_3, pero si por el aumento de la disponibilidad de N (Bassin et al 2007). Wyness et al.,

(2011) observaron un efecto modulador del N en la biomasa aérea de plantas de *Dactylis glomerata* expuestas a concentraciones elevadas de O_3, pero sin efecto en la biomasa subterránea. A escala de ecosistema, es importante destacar que en plantas expuestas a concentraciones elevadas de O_3, a comienzo del otoño, el contenido de N foliar es mayor debido a una reducción de la reabsorción (previa a la caída de la hoja) provocada por el contaminante, un efecto que puede alterar la liberación de nutrientes al suelo por descomposición de la hoja y con ello al ciclo biogeoquímico del N (Uddling et al., 2006; Lindroth et al., 2001).

En la actualidad en amplias zonas rurales de la península Ibérica, donde la dehesa forma parte de su paisaje, es frecuente el registro de niveles elevados de O_3 de forma crónica (Notario et al., 2012, 2013), junto con valores medios de depósito de N (en un rango de 10-38 kg ha^{-1} año) (Sanz et al., 2002; Rodá et al., 2002; García-Gómez et al., 2014). En ambos casos, se sobrepasan los umbrales de daño definidos en base al CLRTAP o a la Directiva de Calidad de Aire para asegurar la salud de la vegetación. Sin embargo, hasta el desarrollo del presente trabajo, no se había realizado ningún estudio sobre los efectos de esta combinación de contaminantes en ninguna comunidad herbácea mediterránea.

1.5. EVALUACIÓN DE LOS EFECTOS DEL OZONO SOBRE LA VEGETACIÓN

1.5.1 Metodologías de estudio

Las distintas técnicas y sistemas experimentales utilizados en el estudio de los efectos de los contaminantes atmosféricos en la vegetación se pueden clasificar en tres grandes grupos: sistemas de análisis en condiciones naturales, seminaturales y controladas. Cada uno de ellos permite responder a diferentes aspectos relacionados con la fitotoxicidad de los contaminantes atmosféricos y presentan diferentes controles sobre las condiciones de desarrollo del experimento.

Los estudios realizados en condiciones controladas (cámaras de fumigación, invernaderos adaptados, etc.), permiten mantener los parámetros climáticos (luz, temperatura, humedad) y las concentraciones de los contaminantes de acuerdo a las necesidades del experimento. Los experimentos pueden ser de este modo reproducibles, por lo que son de gran interés para el análisis de efectos a nivel bioquímico y fisiológico.

Los sistemas basados en condiciones seminaturales o sistemas de exposición en campo, pueden eliminar un contaminante o añadir una fracción controlada del mismo. Aunque dentro de estos se incluyen diversos tipos, el más difundido son las Cámaras de Techo Descubierto (OTCs, *Open Top Chambers*), junto a los sistemas de fumigación a cielo abierto. Las primeras modifican levemente las condiciones microclimáticas, pero presentan la ventaja de que permiten un mejor control de las exposiciones. Las OTCs son muy utilizadas en lugares donde las velocidades del viento desaconsejan la utilización de sistemas de fumigación a cielo abierto al impedir un control adecuado de los niveles del contaminante. El sistema experimental de OTCs ha sido el empleado para el estudio de la sensibilidad de especies pascícolas mediterráneas anuales que se presenta en esta memoria.

En los estudios realizados en condiciones naturales, las plantas se exponen al aire ambiente sin ningún tipo de cámaras o sistemas de exclusión de aire, de forma que están sometidas a las variaciones climáticas naturales y a las concentraciones ambientales de los contaminantes atmosféricos. Dentro de estos estudios se incluyen los análisis mediante bioindicadores/biomonitores, los sistemas que emplean algún agente o sustancia química que protege a las plantas de los efectos que provoca el contaminante y los análisis de gradientes. Son sistemas cuya puesta en marcha es sencilla y económica, por lo que son empleados con cierta frecuencia para la caracterización de la extensión e intensidad de daño que puede sufrir una zona afectada por un contaminante, especialmente si éste tiene un carácter regional como el O_3.

Los bioindicadores son plantas muy sensibles a un contaminante que desarrollan síntomas foliares visibles específicos al mismo, lo que permite hacer estudios sobre la extensión de un problema regional de contaminación. Los biomonitores son plantas sensibles que desarrollan síntomas visibles específicos a un contaminante y cuya cuantificación sirve para caracterizar la concentración ambiental del contaminante. De entre los agentes químicos protectores de los daños que sufren las plantas por los contaminantes, el más empleado para evitar los efectos del ozono es el etilén-diurea (EDU). El sistema EDU/NO EDU se basa en determinar la respuesta diferencial (desarrollo de síntomas, producción, biomasa etc.) entre plantas expuestas al ambiente sin EDU y plantas expuestas a las mismas condiciones pero a las que se les ha añadido el EDU como agente protector al O_3 y que por tanto ejercen de plantas control.

En la actualidad el estudio de los efectos de la contaminación en la vegetación en gradientes de contaminación está siendo muy utilizado. La presencia de un gradiente de concentración de un contaminante en una zona, permite el análisis del desarrollo y estado de salud de la vegetación natural expuesta a los distintos niveles del contaminante; aunque es necesario para la correcta interpretación de los datos obtenidos, tener en cuenta la posibilidad de que coexistan otros gradientes naturales climáticos o edáficos que puedan modificar la respuesta de las plantas al contaminante.

1.5.2. Descripción de la instalación de Cámaras de Techo Descubierto

En este apartado se describe en detalle la instalación de OTCs utilizada en los distintos experimentos realizados en la presente memoria. Se puede obtener información más detallada en Pujadas *et al*. (1997). Se emplearon OTCs tipo NCLAN (*National Crop Loss Assesment Network*). El NCLAN fue un programa diseñado por la US EPA (*United States, Environmental Protection Agency*) y desarrollado en el período de 1980-1987, para la valoración económica de los efectos de los contaminantes atmosféricos en los principales cultivos agronómicos de los Estados Unidos. Se normalizaron las metodologías de estudio de los efectos en vegetación, las técnicas de análisis y los criterios de calidad de los datos.

El campo experimental se ubicó en la finca MIGJORN S.A. (Delta del Ebro, Tarragona; 40º 41.5´ N, 0º 48´ E), constando de 12 parcelas circulares de 3 metros de diámetro donde se establecieron cuatro tratamientos de O_3, con 3 réplicas por tratamiento. En tres tratamientos se emplearon OTCs, mientras que las tres parcelas restantes permanecieron sin cámaras, actuando así como control para el análisis del "efecto cámara"; a estas parcelas se las denominó ambientales (AA). Las parcelas encerradas en OTCs estaban adscritas a los siguientes tratamientos: Aire Filtrado (CFA), Aire No Filtrado (NFA) y Aire No Filtrado suplementado con O_3 (NFA+). En las parcelas CFA se utilizó carbón activo para eliminar el O_3, con una eficacia teórica de filtración de este contaminante del 90%. En los tratamientos NFA, los niveles de O_3 reprodujeron los valores ambientales de la finca. Por último, al tratamiento NFA+ se añadió un suplemento de O_3 a los niveles ambientales para conseguir reproducir una atmosfera con niveles de O_3 cercanos a los máximos horarios que se registran en la península (incremento de unos 40 ppb durante las horas centrales del día). La disposición de las parcelas de los distintos tratamientos se realizó siguiendo una distribución de bloques al azar para eliminar la posible variabilidad debida a la situación de la parcela. Las cámaras se situaron en filas en dirección este-oeste a una distancia adecuada para evitar el sombreado entre las cámaras.

Figura 5. Instalación Experimental de Cámaras de Techo Descubierto (*Open Top Chambers, OTCs*), del grupo de Ecotoxicología de los Contaminantes Atmosféricos (Departamento de Medio Ambiente, CIEMAT), en La finca La Higueruela/CSIC (Santa Olalla, Toledo) (Autor: J.M. Gómez Camacho).

Las OTCs empleadas se construyeron con barras de aluminio formando un cilindro de tres metros de diámetro, rematado en su parte superior por un cono truncado que evita la entrada de aire a través de la abertura superior (alcanzando una altura máxima de 2.9 m). La mitad superior de la cámara está cubierta por un plástico de una sola lámina de polietileno transparente; mientras que la mitad inferior se cubre con plástico de doble lámina, cuya lámina interior presenta perforaciones circulares que permiten el flujo de aire hacia el interior de la cámara (Figura 6). La cubierta inferior se conecta con un motor, protegido por una caja metálica, que impulsa aire al interior de la doble lámina; con este sistema el aire es repartido uniformemente por todo el perímetro de la cámara y, siguiendo un flujo vertical, es expulsado por la abertura superior de la misma. Salvo velocidades altas de viento en el exterior que producirían turbulencias que invertirían el flujo descrito, el funcionamiento normal de las cámaras impide la entrada del aire ambiente en su interior.

El aire procedente de las OTCs y parcelas AA se muestreó cada 10 minutos de forma secuencial para analizar las concentraciones de O_3, SO_2 y NO_x. Se utilizaron los siguientes monitores automáticos: para el O_3 se utilizó el modelo 1008 RS (Dasibi), cuyo método de medición es fotometría ultravioleta; para el SO_2 se utilizó el modelo 4108 (Dasibi), cuya técnica de medida es fluorescencia ultravioleta; y para los óxidos de nitrógeno se utilizó el modelo 2108 (Dasibi), cuyo método de medición se basa en reacciones de quimioluminiscencia. El aire se recogía de cada parcela a la altura del dosel vegetal y se dirigía a la cabina de control mediante un tubo de teflón empleando una bomba de aspiración común para todas las líneas.

Se registraron las condiciones meteorológicas en el interior de 4 parcelas, cada una correspondiente a un tratamiento de O_3, empleando sensores para radiación solar fotosintéticamente activa (PAR), temperatura y humedad del aire. Los sensores empleados fueron: para el PAR (Skye Instruments, Mod. SKP 210), para la temperatura (Skye Instruments, Mod. SKH 2013) y para la humedad relativa (Skye Instruments, Mod. SKH 2010). Los sensores se ubicaron a la altura del dosel vegetal para coincidir también con el punto de muestra del aire.

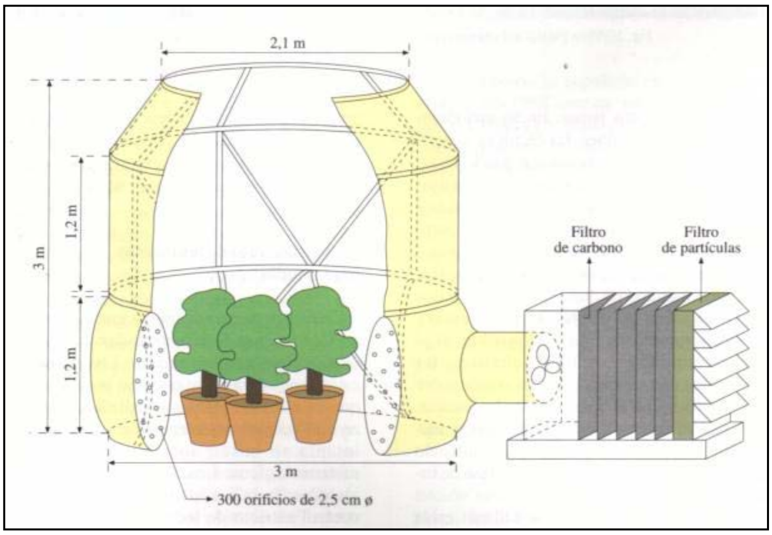

Figura 6. Esquema de cámara de techo descubierto tipo NCLAN modificada (Adaptada de Heagle et al., 1979).

1.5.3. Índices para la evaluación del riesgo de daños por ozono. Niveles críticos

Un elemento fundamental en el estudio de los efectos de los contaminantes atmosféricos consiste en la cuantificación de la relación entre el contaminante y la respuesta biológica, lo que requiere el empleo de índices de exposición. En líneas generales se pueden distinguir tres generaciones de índices utilizados para el establecimiento de niveles críticos para la vegetación en el marco del CLRTAP: índices de exposición basados en promedios, índices de exposición basados en la concentración acumulada por encima de un nivel de fondo (AOT), índices de dosis absorbida de ozono por vía estomática (POD_y).

Los índices de exposición basados en promedios fueron los primeros establecidos (1988). Se basan en el cálculo de medias de concentración del contaminante en aire durante periodos de tiempo determinados (1, 10, 12 o 24 horas). El problema más importante de este tipo de índices es que no distinguen entre las diferentes distribuciones de concentración: concentraciones elevadas del contaminante durante espacios cortos de tiempo pueden presentar el mismo promedio que concentraciones moderadas mantenidas de forma más constante. De esta forma, se asume que todas las concentraciones incluidas en el promedio tienen el mismo efecto sobre las plantas, cuando la realidad puede ser diferente.

Los índices de concentración acumulada se basan en la suma de concentraciones de O_3 (la terminología europea para este tipo de índices es AOT, *Accumulated exposure Over a given Threshold)*, por encima de un determinado umbral, para un período de tiempo establecido. Actualmente el umbral de 40 ppb es el que se utiliza dentro del CLRTAP y en la legislación europea (Directiva 2008/50). Este índice se utiliza para la cuantificación de los efectos mediante las funciones exposición-respuesta, que dieron lugar a la primera generación de niveles críticos (NCs) basados en la concentración acumulada en aire del contaminante (CL_{ec}) en 1996 (Kärenlampi y Skärby, 1996; Fuhrer *et al.*, 1997).

Aunque los índices basados en la exposición acumulada se relacionan mejor con los efectos que los índices basados en promedios, no contemplan la influencia de determinados parámetros ambientales y biológicos que pueden modular la toxicidad del O_3. Las concentraciones más elevadas de O_3 en ambiente, pueden no estar asociadas con los mayores flujos de entrada al interior de la planta y por tanto con un mayor efecto (Fuhrer *et al.*, 1997). Esto sucede por ejemplo, cuando el déficit hídrico limita la conductancia estomática, y por tanto, el flujo del contaminante al interior vegetal. En la última década se han desarrollado funciones de dosis-respuesta, considerando el cálculo de los flujos de O_3 al interior vegetal. A partir de estas funciones se han generado una nueva generación de NCs basados en flujos de absorción (CL_{ef}). El cálculo de este tipo de índices (PODy, *Phytotoxic Ozone Dose*) requiere de un importante esfuerzo experimental, que permita la modelización del comportamiento estomático de las distintas especies para el cálculo de la cantidad de contaminante que penetra al interior vegetal. Esta metodología se ha desarrollado en el marco del CLRTAP mediante modelos específicos que cuantifican el depósito de O_3 total en la superficie vegetal, siendo el absorbido por los estomas su componente principal. Los efectos que provoca el O_3 en la vegetación, están en general más directamente relacionados con este depósito seco de O_3, que con la concentración en aire del contaminante.

1.5.4. Depósito seco sobre la vegetación

Como ya se ha comentado anteriormente, se considera depósito seco de O_3 sobre la vegetación tanto el que se absorbe a través de los estomas, siguiendo el proceso de intercambio gaseoso habitual de la planta, como el que reacciona con las superficies vegetales (cutícula de tallos y hojas), pero cuantitativamente el primer proceso es dominante. Por ello, aunque el cálculo del depósito seco depende de propiedades de la superficie vegetal y de las características propias del gas, son muy importantes las variables meteorológicas de las que depende la apertura estomática que determina el flujo de O_3 al interior vegetal, como la temperatura, el déficit de vapor de agua, la disponibilidad de agua en el suelo y la radiación solar (González-Fernández et al., 2010).

El comportamiento de entrada del O_3 en la hoja es similar al de otros gases, y depende de la constante de difusión del gas en el aire, que es directamente proporcional al gradiente de concentraciones entre el interior y el exterior celular, e inversamente proporcional a una serie de resistencias que se oponen al movimiento de las moléculas. Por lo tanto, la entrada del O_3 en la planta puede explicarse mediante un análogo a un sistema de resistencias esquematizado en la Figura 7.

La primera resistencia es la aerodinámica (R_a) que determina el paso de una molécula desde la atmósfera hasta la capa justo en contacto con la superficie de la vegetación. Este proceso depende de factores como la altura y la estructura del dosel, la velocidad del viento o la rugosidad de la superficie. A continuación la resistencia de la capa límite (R_b), determina la concentración de O_3 en la capa de aire justo en contacto con la superficie, susceptible de reaccionar con la misma, que varía con la velocidad del viento y parámetros morfológicos de la hoja. Finalmente, la fracción de la concentración de O_3 que realmente entra en contacto con la superficie está determinada por la resistencia de superficie (R_c) que presenta dos componentes, la resistencia cuticular (R_{ext}), que determina la tasa de reacción del O_3 con la superficie de la cutícula y la resistencia a la absorción estomática (R_s) que a su vez está controlada por la apertura estomática. Esta absorción se encuentra principalmente regulada por el grado de apertura de los estomas o conductancia estomática (g_s), calculada como el inverso de R_s (Emberson *et al.*, 2000a). La conductancia estomática está controlada por multitud de factores climáticos (temperatura del aire, radiación solar, humedad del aire y del suelo, etc.) y fisiológicos (fenología, edad de la hoja, presencia de hormonas vegetales, estado hídrico de la planta, etc.), que determinan el grado de apertura o cierre de los estomas y, por lo tanto, la velocidad del intercambio de gases.

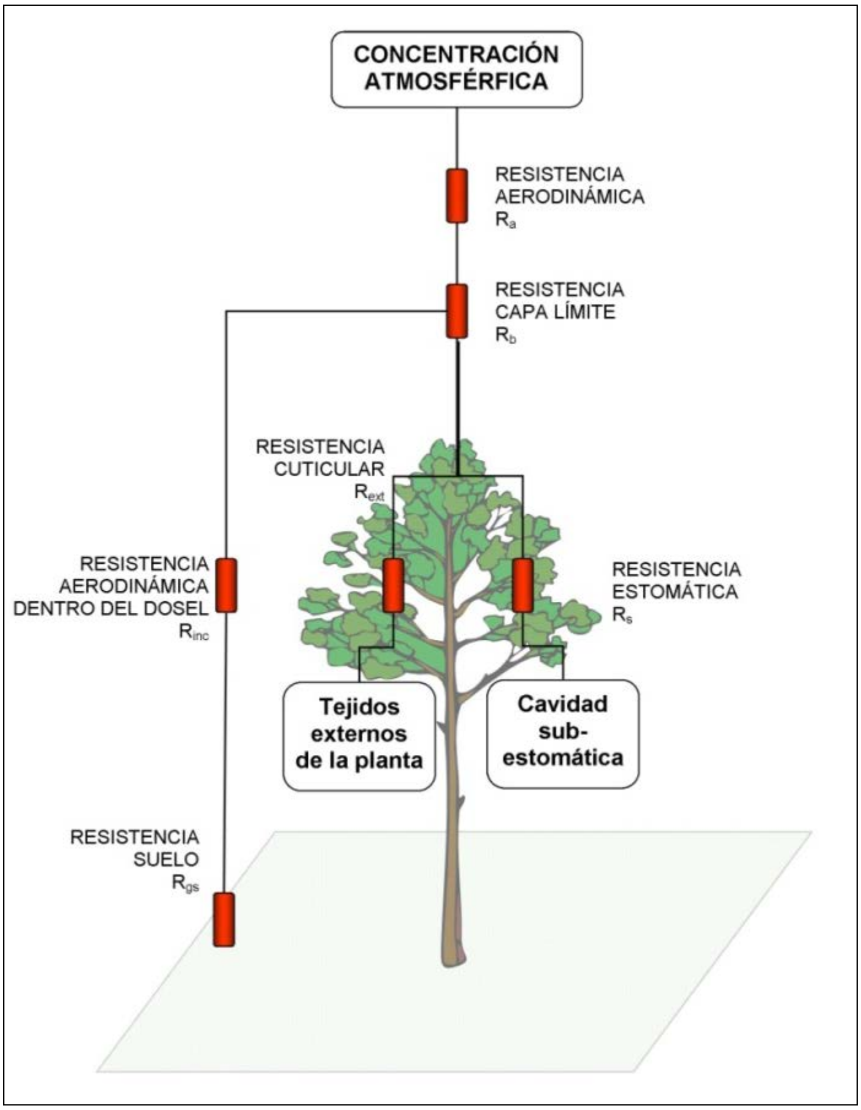

Figura 7. Modelo de resistencias para el cálculo del depósito seco de ozono sobre la vegetación (Adaptada de CLRTAP, 2010).

1.5.5 Cuantificación del depósito seco de ozono sobre la vegetación

Dentro del modelo de dispersión de la contaminación atmosférica del EMEP (*European Monitoring and Evaluation Programme*), el módulo DO_3SE (*Deposition of Ozone and Stomatal Exchange*) representa el proceso de depósito seco del O_3 utilizando el modelo de resistencias (Gaastra, 1959; Emberson *et al.*, 2000b; Simpson *et al.*, 2003, 2007). Según este esquema, el depósito de O_3 desde la atmósfera atraviesa una serie de sumideros (que se corresponden con las resistencias vistas anteriormente) que reducen su concentración (Grünhage *et al.*, 2004). El flujo que recorre una molécula desde que se encuentra en la atmósfera hasta que se deposita sobre una superficie es expresada como el múltiplo de la concentración de O_3 y el inverso de la resistencia para ese paso concreto. La tasa de depósito se expresa como una velocidad de flujo vertical de depósito en unidades de espacio x tiempo^{-1}.

Flujo = $[O_3] \cdot 1/R$

Donde R es la suma de todas las resistencias (aerodinámica, capa límite, cuticular, estomática, del dosel y del suelo). Cada uno de los procesos contemplados por el modelo de resistencias se representa en la figura 7.

Dentro del módulo DO_3SE, la modelización de la conductancia estomática se basa en el modelo multiplicativo inicialmente propuesto por Jarvis (1976) y modificado por Emberson *et al.* (2000a). Este modelo permite el cálculo del flujo de O_3 absorbido, susceptible de provocar daños sobre la vegetación (Pleijel *et al.*, 2007). Esta aproximación está basada en el "*big leaf approach*" descrito por Jarvis (1976), según la cual el dosel sobre el que se deposita el O_3 se representa como una superficie horizontal uniforme. En el modelo multiplicativo, la conductancia se calcula como el producto de la conductancia máxima (g_{max}) y una serie de parámetros que la corrigen, basados en variables ambientales y particulares de cada especie que actúan de forma independiente como con la temperatura, el déficit de presión de vapor (VPD), la radiación solar o la disponibilidad de agua en el suelo (Danielsson *et al.*, 2003). El efecto de esos parámetros sobre g_{max} se determina utilizando curvas envolventes: líneas que conectan la parte externa de las nubes de puntos en gráficos que enfrentan la conductancia estomática relativa frente las diferentes variables ambientales (luz, temperatura, etc.) durante la medición de dicha conductancia. La Figura 8 representa un ejemplo de este tipo de gráficos.

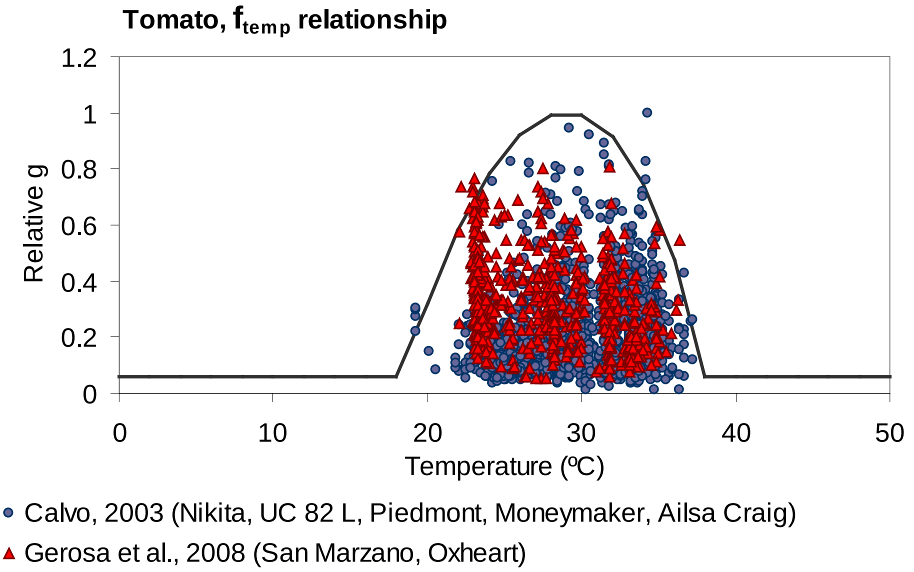

Figura 8. Gráfica de curva envolvente de conductancia estomática relativa y temperatura (Fuente: Adaptado de González-Fernández et al., 2014).

Se considera que estas líneas describen la dependencia funcional entre los dos parámetros que se representan, e indican por tanto la conductancia máxima potencial para un valor dado de cada variable (Jarvis, 1976; Emberson *et al.*, 2000a; Schmidt *et al.*, 2000; Danielsson *et al.*, 2003). A partir de ellas se parametriza la siguiente ecuación que representa el modelo multiplicativo utilizado por DO$_3$SE (Pleijel *et al.*, 2007; UNECE, 2008).

$$g_s = g_{\max} * [\min(f_{phen}, f_{O3})] * f_{light} * \max[f_{\min}, (f_{temp} * f_{VPD} * f_{SWP})]$$

Donde g_s es la conductancia estomática (mmol H_2O m^{-2} s^{-1}) y g_{max} es la conductancia estomática máxima que se modifica por el efecto del resto de parámetros ambientales y fisiológicos representados por f_{phen}, f_{O3}, f_{light}, f_{temp}, f_{VPD} y f_{SWP}, para fenología (estadio del desarrollo), ozono, luz solar, temperatura, déficit de presión de vapor del aire y humedad del suelo respectivamente, todos ellos considerados como determinantes en la regulación de la apertura estomática según Emberson *et al.* (2000a). Estas funciones varían entre 0 (representa cierre estomático) y 1 (representa apertura total de los estomas). Toda esta metodología está desarrollada en el manual técnico del Convenio (CLRTP, 2010; www.icpmapping.org).

1.5.6 Legislación de los niveles críticos de vegetación seminatural en el marco del CLRTAP y de la Unión Europea

La necesidad de establecer un programa conjunto a nivel internacional para desarrollar políticas y estrategias de gestión medio ambiental para el control de la contaminación atmosférica, fue el origen del Convenio de Naciones Unidas sobre Contaminación Atmosférica Transfronteriza a Gran Distancia (*Convention on Long-Range Transboundary Air Pollution*, CLRTAP/UNECE). El CLRTAP es la organización internacional encargada de generar los protocolos sobre reducción de emisiones de contaminantes, que a su vez, deben ser reflejados por los países firmantes mediante decretos específicos. Entre los objetivos y compromisos adquiridos al formar parte del CLRTAP, están limitar y reducir la contaminación atmosférica transfronteriza, revisar periódicamente las políticas y estrategias nacionales de reducción de contaminantes y colaborar con el programa EMEP (*European Monitoring and Evaluation Programme*) sobre seguimiento en continuo y evaluación del transporte atmosférico de contaminantes (http://www.unece.org/env/lrtap/welcome.html). El CLRTAP se suscribió en 1979 en el ámbito del Consejo Económico para Europa de Naciones Unidas (UNECE), entró en vigor en 1983 y fue ratificado por 34 países y la Unión Europea con entidad propia. Actualmente está constituido por 51 miembros (entre ellos los países de la Unión Europea, incluida España, y la propia UE). Dentro del organigrama del Convenio se encuentra el Grupo de Trabajo sobre Efectos (*Working Group on Effects,* WGE), responsable de ofrecer información acerca de los efectos de los contaminantes atmosféricos sobre la salud humana, el medio ambiente y los materiales, con el objeto de establecer la base científica que sirva de referencia para la toma de decisiones sobre políticas medioambientales. Las actividades del WGE se llevan a cabo mediante el establecimiento de Programas de Cooperación Internacional (*International Cooperative Programs*, *ICPs*). La evaluación y control de los efectos de la contaminación atmosférica en la vegetación se lleva a cabo en el *ICP-Vegetation* (*Internacional Cooperative Programme on Effects of Air Pollution on Natural Vegetation and Crops*); ICP *Forests* e *ICP-Integrated Monitoring*.

El CLRTAP adoptó la metodología de niveles/cargas críticas para establecer los protocolos de reducción de contaminantes. El nivel/carga crítica se define como la concentración/depósito umbral por encima del cual aparecen efectos perjudiciales para los receptores sensibles. Basándose en esta aproximación, las reducciones de las emisiones de los contaminantes o de sus precursores se realizarán con el fin de conseguir que las concentraciones sean inferiores a esos niveles o cargas críticas. El establecimiento de estos niveles críticos para cada contaminante se revisa de forma continua con el objetivo de incorporar los últimos avances científicos (CLRTAP, 2010). Los niveles críticos establecidos para los distintos contaminantes y receptores se consensuan en los grupos de trabajo del Convenio de acuerdo al conocimiento científico del momento y constituyen la base sobre la que se definen las directivas europeas de calidad del aire.

La metodología para el cálculo de los niveles críticos (NCs) y cargas críticas, se describe en el manual técnico del Convenio: *Mapping Manual* (*Manual on Methodologies and Criteria for Modelling and Mapping Critical Loads and Levels and Air Pollution Effects, Risks and Trends; http://www.icpmapping.org/Mapping_Manual;* CLRTAP, 2010), cuyo contenido se revisa periódicamente. Se han establecido tres tipologías de vegetación: especies agrícolas y hortícolas, especies arbóreas (bosques) y vegetación seminatural. Dentro de este último tipo de vegetación, que incluye pastos y pastizales de forma general, se han considerado distintos grados de manejo para su catalogación: pastos productivos, muy manejados y de alto rendimiento; pastos con alto valor de conservación, de bajo manejo y con muy poco aporte de fertilizantes; y pastos naturales. Además, también se considera la temporalidad de la comunidad en la clasificación: comunidades dominadas por especies perennes o anuales.

La vegetación herbácea (seminatural) está constituida por un número muy elevado de especies, en el rango de 4000 especies sólo en Europa, y comunidades, lo que complica mucho la definición de NC para este grupo de vegetación tan amplio y de sensibilidad al O_3 tan desconocida (menos del 5% de las especies han sido estudiadas).

Como se ha comentado en el apartado anterior, existen dos aproximaciones para la determinación de los NC: la que se basa en índices que reflejan la concentración en aire del contaminante, tipo AOT40; y la que se basa en el flujo de O_3 real absorbido por la vegetación, con el empleo de los índices POD_y. Estos últimos más recientes, se han desarrollado de forma paralela a la disponibilidad de modelos específicos para el cálculo del flujo estomático de O_3 descrito en el apartado anterior mediante el modelo DO_3SE.

Para las comunidades perennes, el NC basado en el índice de exposición acumulada AOT40 (CL_{ec}) es de 5 ppm h acumulado durante un periodo de 6 meses

(Tabla 1.1). Este valor límite se ha calculado considerando un máximo de efecto del 10% en la biomasa (pérdida del 10%). Los niveles críticos basados en flujo de O_3, utilizan como especie representativa de los pastos perennes a *Trifolium repens* para la modelización de la dosis absorbida mediante el DO_3SE. Esta especie ha sido seleccionada por su alta sensibilidad al O_3 en parámetros de biomasa y calidad pascícola (Tabla 1.2). En base a ella se ha establecido un nivel crítico basado en flujo de O_3 absorbido (POD_1) de 2 mmol m^{-2} PLA (*Projected leaf área*).

Para las comunidades dominadas por especies anuales, el nivel crítico basado en el índice de exposición AOT40 (CL_{ec}) se estableció de forma provisional en 3 ppm h considerando un período acumulado de 3 meses o la estación de crecimiento, y permitiendo un efecto máximo del 10% en biomasa y/o producción de semilla. Este valor se estableció de forma provisional, al no estar disponibles funciones de respuesta para este tipo de comunidad. No existían, hasta el desarrollo del presente trabajo, NCs basados en flujos (CL_{ef}) para este tipo de comunidad. En esta memoria, se revisan y se proponen nuevos CL_{ec} y CL_{ef} para comunidades anuales en base a la información experimental obtenida durante el desarrollo del estudio.

Receptor	**Efecto (10 % de reducción)**	**Índice**	**Nivel crítico** (ppm h) (periodo de cálculo)
Pastos anuales	Reducción de crecimiento y/o producción de semilla	AOT40	**3** (3 meses o ciclo de crecimiento si es más corto)
Pastos perennes	Reducción de crecimiento	AOT40	**5** (6 meses)

Tabla 1.1. Niveles críticos de ozono para vegetación seminatural basados en concentración de ozono en aire (índice AOT40) (CLRTAP, 2010).

Receptor (especie representativa)	**Efecto (% de reducción)**	**Índice**	**Nivel crítico** (mmol m^{-2} PLA)
Pastos productivos perennes (*Trifolium repens*)	Reducción biomasa (10%)	POD1	**2**
Pastos de conservación perennes (*Trifolium repens*)	Reducción biomasa (10%)	POD1	**2**
Pastos de conservación perennes (provisional, *Viola* ssp.)	Reducción biomasa (15%)	POD1	**6**

Tabla 1.2. Niveles críticos de ozono para vegetación seminatural basados en dosis absorbida de ozono (índice PODs) (CLRTAP, 2010).

Los niveles críticos basados en concentración (CL_{ec}) del CLRTAP han sido la base de los valores objetivo establecidos en la Directiva Europea de Calidad del Aire (2008/50/CE) para la protección de la vegetación. En esta Directiva no se detallan tipos de vegetación y se considera solo el índice de exposición acumulada AOT40 (entre mayo-julio). De acuerdo con esta Directiva, para cumplir con los valores objetivo (a cumplir desde 2010), el valor del índice AOT40 entre mayo-julio no debe superar el valor de 9 ppm h (18.000 µg m^{-3} h) promediado durante un período de 5 años. Como valor objetivo a largo plazo, sin fecha de cumplimiento definida, el índice AOT40 no debe superar los 3 ppm h (6.000 µg m^{-3} h).

El establecimiento de los umbrales de daño de los contaminantes atmosféricos es un proceso dinámico sometido a revisión periódica con el fin de incorporar los últimos conocimientos científicos. Los trabajos presentados en esta memoria han contribuido y contribuyen a los debates para el establecimiento de los niveles críticos para vegetación seminatural de comunidades dominadas por especies anuales. Los NC propuestos en el último capítulo serán presentados en la próxima reunión anual del Grupo de Trabajo sobre Vegetación (*Internacional Cooperative Programme on Effects of Air Pollution on Natural Vegetation and Crops, ICP-Vegetation*) del CLRTAP/UNECE para su aprobación e inclusión en los manuales técnicos del Convenio, con la finalidad de ser empleados en los análisis de riesgo de los efectos del O_3 para este tipo de vegetación en el ámbito mediterráneo.

1.6. LA DEHESA

1.6.1. Introducción

En amplias zonas de la península Ibérica, mayoritariamente localizadas al suroeste, y por lo general no adecuadas para la actividad agrícola, la actuación del hombre en el bosque mediterráneo ha dado origen al sistema agrosilvopastoral “dehesa”, formado por un dosel arbóreo disperso bajo el cual se desarrollan las comunidades de pastos que sirven de alimento a la fauna y la ganadería (San Miguel, 1998). La dehesa aparece siempre en zonas llanas, penillanuras o suaves cerros, y presenta dos características ambientales que dan sentido a su sistema de gestión. En primer lugar un clima marcadamente mediterráneo, con veranos secos acompañados de un fuerte período de sequía; y con un invierno muy frio que también conlleva parada vegetativa (Roig et al, 2013). En segundo lugar, sus suelos, mayoritariamente oligotrofos, son pobres en nutrientes, particularmente en fósforo y calcio, lo que los convierten en poco adecuados para una agricultura sostenible. Sin embargo, los productos directos obtenidos de la ganadería son los más importantes de la dehesa. Además, el ganado es la herramienta fundamental para la conservación y mejora de los pastos: dispersa las semillas, fertiliza, aumenta la materia orgánica del suelo etc. La buena gestión ganadera es fundamental para la dehesa, sin embargo puede ser su mayor peligro si no se gestiona adecuadamente, ya que la regeneración del arbolado puede ponerse en peligro con cargas ganaderas excesivas o si no se acotan zonas al ganado para este regenerado (Olea & San Miguel, 2006). Desde mediados del siglo pasado, la caza ha comenzado a ser una actividad económica notable de la dehesa, con una capacidad de generación de ingresos a sus propietarios mayores que las actividades tradicionales; el resultado ha sido un incremento de la densidad de fauna herbívora, especialmente ungulados (*Cervus elaphus hispanicus, Sus crofa*), que están creando serios problemas de sostenibilidad del sistema.

El equilibrio obtenido por el hombre entre producción y conservación de la naturaleza, así como la gran diversidad biológica producto de la integración de los distintos compartimentos que aparecen en la dehesa, hacen de ella un ejemplo de gestión sostenible (García et al., 2014). Por estas razones, la dehesa está protegida por la Directiva Hábitat 92/43/EEC e incluida en la red Natura 2000.

Figura 9. Dehesa de encina (*Quercus ilex*) en Talarrubias (435 m altitud, Badajoz). (Autor: J. Sanz).

1.6.2. El arbolado

La dehesa tiene una estructura de bosque abierto, cuyos árboles tienen un papel fundamental en el funcionamiento y estabilización del sistema, proporcionando una gran variedad de beneficios indirectos y directos. Las especies arbóreas principales que forman las dehesas pertenecen al género *Quercus*: entre las especies esclerófilas y perennes destacan *Quercus ilex* y *Quercus suber*; entre las caducifolias *Quercus faginea* y *Quercus pyrenaica*. La densidad del arbolado es variable, considerándose entre 20 y 100 pies/hectárea como los valores más adecuados. Dependiendo de la densidad y de la edad del arbolado, un parámetro de gran importancia es la fracción de cabida cubierta, que oscila en sus mejores valores entre el 10 y 50 %. Entre sus funciones indirectas está la protección contra la erosión, la modulación del ciclo hidrológico, la fijación de carbono y la extracción de nutrientes de horizontes inferiores. Desde un punto de vista de la biodiversidad, sirve de refugio y muchas veces de lugar de cría de gran cantidad de animales de gran valor ecológico, y además las características especiales que crea bajo sus copas aumentan la diversidad de las especies vegetales. Del arbolado de la dehesa también se pueden obtener una gama de beneficios directos como el carbón, el corcho, y alimento mediante ramón o mediante su fruto, la bellota, un alimento de gran aporte nutricional y cuyas producciones son muy variables debido a la vecería de los *Quercus*. El árbol es fundamental en la dehesa y por lo tanto la gestión de la misma ha de ir encaminada a la protección y sobre todo a su regeneración. En la actualidad, uno de los mayores problemas de la dehesa es la falta de regeneración debido al abandono de la trashumancia, la sustitución del ganado ovino por vacuno y al incremento de las cargas ganaderas debido a las ayudas generadas por la política agraria de la Unión Europea. En la década de los ochenta del siglo pasado apareció una enfermedad fúngica agravada con otros estreses abióticos y bióticos que provoca la muerte de árboles adultos (San Miguel, 1998).

1.6.3. Arbustos y matorrales

En las dehesas el estrato arbustivo es escaso, ya que su finalidad principal es tener la mayor superficie destinada al pasto. Sin embargo, es muy necesario que existan pequeñas manchas de matorral y arbustos permanentes, ya que sirven de refugio y alimento a la fauna y especialmente a aquella que ayuda a la diseminación de la bellota y por lo tanto a la regeneración del arbolado. La presencia de arbustos y matorrales incrementa la biodiversidad, con especies típicas del sotobosque mediterráneo: *Genista*, *Ulex*, *Ononis*, *Cytisus*, *Retama*, *Pistacia*, *Lonicera*, *Asparagus*, *Juniperus*, *Rosa*, *Crataegus*, *Berberis*, *Erica*, *Lavandula*, *Rosmarinus*, *Thymus*, *Helianthemum*, *Teucrium*, *Cistus*, *Halimium*, etc. (San Miguel, 1998).

1.6.4. Los pastos naturales

El principal objetivo de la dehesa es la alimentación de la ganadería extensiva mediante los pastos. Las características particulares del clima mediterráneo son la causa de que estos pastos estén mayoritariamente integrados por especies anuales que pasan el periodo de sequía en forma de semilla (Peco et al., 2009). Pero también aparecen especies perennes en aquellas zonas con mejor calidad del suelo favorecidas por la gestión del pastoreo y zonas de mayor humedad edáfica debido a su orografía y orientación. Existe una gran variabilidad espacial y temporal en producción y calidad forrajera de estos pastos relacionada con la variabilidad de las condiciones meteorológicas del clima mediterráneo (Vázquez de Aldana et al., 2008).

El objetivo de la gestión de los pastos naturales de la dehesa para su mejora, se encamina a incrementar la cantidad de leguminosas, que son las especies que aportan más cantidad de materia nitrogenada, y hacia un pastoreo racional con una carga ganadera adecuada; aunque debido a la existencia de los periodos estacionales críticos de invierno y verano, siempre es necesario la aportación al ganado de suplementos alimenticios externos a la dehesa o generados en ella en época de bonanza (San Miguel, 2001).

Las principales comunidades de pastos que habitan en las dehesas son las siguientes:

1.6.4.1. Pastizales terofíticos acidófilos sobre suelos no arenosos (Helianthemetalia)

Básicamente se encuentra constituido por especies herbáceas anuales de talla pequeña a mediana. Presentan una cobertura incompleta del suelo (50-80%), con un máximo productivo en primavera y después en otoño. Presentan escasez de leguminosas, dominando las gramíneas anuales: *Briza maxima*, *Molineriella laevis*, *Micropyrum tenellum*, *Psilurus incurvus*, *Vulpia myuros*, etc. Entre las leguminosas más características: *Ornithopus compressus*, *Trifolium arvense*, *Trifolium campestre*, *Trifolium cherleri.* Puede ser muy abundante la cistácea anual *Xolantha guttata* (*Tuberaria guttata*). Una buena gestión del ganado puede hacer que esta comunidad evolucione hacia al majadal o se estabilice en una situación intermedia entre ambos, en ambos casos con más productividad y mayor calidad. Su producción es media-baja (700-1700 kg de MS ha^{-1} año^{-1} (materia seca) (Rivas Goday & Rivas Martínez, 1963; San Miguel, 2001).

1.6.4.2. Pastizales terofíticos de arenales (Malcomietalia)

Están constituidos mayoritariamente por gramíneas anuales de pequeña talla, de escasa calidad nutritiva y fenología temprana. Presentan una cobertura muy baja, alrededor del 50%, y es muy común la presencia de leñosas heliófilas como cistáceas y labiadas de carácter pionero. Entre las gramíneas destacan: *Corynephorus fasciculatus*, *Vulpia ciliata*, *Desmazeria rigida* y entre las leguminosas *Ornithopus compressus*, *Anthyllis lotoides* y *Anthyllis cornicina*. Debido a su fenología temprana, su producción se concentra a finales del invierno y comienzos de la primavera. Su producción es muy baja, unos 300-600 kg de MS ha^{-1} año^{-1} (Rivas Goday & Rivas Martínez, 1963; San Miguel, 2001).

1.6.4.3. Pastizales terofíticos nitrófilos (Thero-Brometalia, Sysimbrietalia)

Los pastizales del orden *Thero-Brometalia* aparecen en terrenos labrados en años anteriores o en aquellos desbrozados. Los suelos tiene un alto contenido en nitrógeno, y en él se desarrollan herbáceas anuales, principalmente gramíneas subnitrófilas. Entre las gramíneas se pueden encontrar: *Aegilops geniculata, Bromus rubens, Lolium rigidum, Taeniatherum caput-medusae*, y las leguminosas: *Medicago orbicularis, Medicago rigidula, Trifolium angustifolium, Trifolium stellattum, Trifolium hirtum*. Su máxima producción es en primavera y después en otoño. La producción estival y de invierno es inexistente La producción es media-alta, en torno a los 2000-3000 kg de MS ha^{-1} año^{-1}. Su calidad es media-baja, muy similar a los pastizales terofíticos normales, siendo aceptable durante el periodo vegetativo. Tienen un bajo contenido en proteína por su escasez de leguminosas.

Los pastizales dentro del orden de *Sysimbrietalia* son ruderales, están dominados por especies anuales con moderadas exigencias en nitrógeno y fosforo. Su aspecto es muy similar a los *Thero-Brometalia* y comparten las familias botánicas que contribuyen a su composición florística así como las posibilidades de aprovechamiento y mejora. Predominan las gramíneas como: *Avena barbata, Bromus hordeaceus, Bromus sterilis, Hordeum murinum*, pero también crucíferas (*Sisymbrium officinale*), geraniáceas (*Erodium ciconium*) etc. (Rivas Goday & Rivas Martínez, 1963; San Miguel, 2001).

1.6.4.4. Majadales (Poetalia)

El majadal es la joya pastoral del medio mediterráneo. Está formado por especies de escasa talla que cubren completamente el suelo con características adaptativas al pastoreo: porte rastrero, bulbos, estolones, sistemas de enterramiento de semillas, etc. Se crean y se sustentan por la gestión del hombre mediante una actuación intensa y continua de altas cargas de ganado, cuyas deyecciones aumentan el contenido orgánico y de nutrientes del suelo. Se debe evitar el laboreo del suelo del majadal, ya que desaparecería rápidamente la materia orgánica oxidable acumulada durante años. La gramínea *Poa bulbosa* es una especie clave, acompañada por otras especies anuales y vivaces de buen valor pascícola. Entre ellas destacan, sobre sustratos silíceos, los tréboles, y muy especialmente, el *Trifolium subterraneum*. La *Poa bulbosa* tiene un agostamiento temprano, al comienzo de la primavera, pero sin embargo su rebrote otoñal es precoz. Desde el punto de vista pastoral, el aprovechamiento se puede adelantar notablemente en otoño con respecto al de los pastos del entorno. El trébol subterráneo tiene un agostamiento tardío y además proporciona alimento con alto contenido de proteína incluso cuando está seco, con lo que se prolonga el periodo de aprovechamiento ganadero del pasto. Su producción es alta, unos 3000 kg de MS ha^{-1} año^{-1}, con buen valor nutritivo (Rivas Goday & Rivas Martínez, 1963; San Miguel, 2001).

1.6.4.5. Vallicares (*Agrostietalia*)

Son pastizales que se encuentran en suelos con humedad edáfica prácticamente durante todo el año, exceptuando el verano. Son vivaces, altos, relativamente densos y estacionales, con agostamiento estival tardío, con una cobertura del 70-100 %. Predominan las gramíneas destacando *Agrostis castellana*, junto a *Festuca ampla, Gaudinia fragilis, Dactylis glomerata, Cynosurus echinatus.* Destaca la escasez de leguminosas, se pueden encontrar entre otras *Trifolium dubium* y *Trifolium campestre*. Florecen a finales de primavera y se agostan a mediados de verano. Su producción es alta, 3000 kg de MS ha^{-1} año^{-1}. Su calidad es media-baja, con baja palatabilidad, con un alto contenido en fibra y escasez de proteína. Estos pastos permiten atenuar la escasez de alimento que se produce una vez que los pastos terofíticos han alcanzado su madurez (Rivas Goday & Rivas Martínez, 1963; San Miguel, 2001).

Las comunidades de pastos a los que podrían aplicarse los resultados obtenidos en esta memoria serían: pastizales terofíticos acidófilos sobre suelos no arenosos (*Helianthemetalia*), pastizales terofíticos de arenales (*Malcomietalia*) y pastizales terofíticos nitrófilos y ruderales (*Thero-Brometalia* y *Sysimbrietalia*).

1.6.5. Mejora de pastos. Pastos artificiales y cultivos

Los objetivos que se persiguen con las actuaciones de mejora de los pastos son: mayor producción en cantidad, especialmente en las épocas más críticas, y mayor calidad bromatológica, con el propósito de aprovechar al máximo los recursos naturales y ahorrar en alimentación suplementaría (Santamaría et al., 2009). Las mejoras deben mantenerse en el tiempo, ya que si son efímeras resultan inadecuadas e inútiles. Los métodos de mejora más frecuentes son: la fertilización fosfórica y la introducción de especies, principalmente leguminosas, destacando el *Trifolium subterraneum* por su buena calidad y su peculiar característica de autosiembra. Se suele acompañar entre otras especies con: *Trifolium striatum, Trifolium cherleri, Trifolium campestre,* *Biserrula pelecinus* y *Ornithopus compressus*. El aumento de leguminosas tiene un doble efecto beneficioso, aumenta la concentración de proteína y permite su utilización en las épocas de escasez (verano y otoño-invierno) como pasto seco. También se utiliza siembras de veza con cereal, normalmente en proporciones de 1-3, para ser consumido a diente o cosechado para su posterior consumo.

El cultivo en la dehesa se realiza normalmente en intervalos entre los dos y cinco años, con el propósito de evitar la invasión de matorral y proporcionar alimento en las épocas críticas para el ganado y la fauna. Los cultivos más frecuentes son cereales: trigo (*Triticum aestivum*), cebada (*Hordeum vulgare*), avena (*Avena sativa*) o triticale (*x Triticum-secale*). El grano es el producto de más valor, que puede ser recogido y aportado el ganado cuanto el pasto no es de suficiente calidad o puede ser directamente pastoreado por el ganado (Santamaría & García White, 2010).

1.7. BIBLIOGRAFÍA

Adame, J.A., Lozano, A., Bolívar, J.P., De la Morena, B., Contreras, J., Godoy, F., 2008. Behaviour, distribution and variability of surface ozone at an arid region in the South of Iberian Peninsula (Seville, Spain). Chemosphere 70, 841-849.

Adame, J.A., Bolívar, J.P., De la Morena, B., 2010. Surface ozone measurements in the southwest of the Iberian Peninsula (Huelva, Spain). Environ. Sci. Pollut. Res. 17 355–368.

Ainsworth, E.A., Yendrek, C.R., Sitch, S., Collins, W.J., Emberson, L.D., 2012. The effects of tropospheric ozone on net primary production and implications for climate change. Annual Review of Plant Biology 63: 637-661.

Alonso, R., Elvira, S., Castillo, F.J., Gimeno, B.S., 1999. Antioxidative defense and photoprotection in Pinus halepensis induced by Mediterranean conditions and ozone exposure. Free Radicals Reseach 31, 59-65.

Alonso, R., Elvira, S., Castillo, F.J., Gimeno, B.S., 2001. Interactive effects of ozone and drought stress on pigments and activities of antioxidative enzymes in Pinus helepensis Mill. Plant Cell and Environment 24(9), 905-916.

Alonso, R., Bermejo, V., Elvira, S., Alfaro, A., Sanz, J., Garraleta, M.H.D., González-Fernández., I., Gimeno, B.S., 2009. La contaminación atmosférica en la Sierra de Guadarrama. Sextas Jornadas Científicas del Parque Natural de Peñalara y del Valle de El Paular

Andersen, C.P., 2003. Source-sink balance and carbon allocation below ground in plants exposed to ozone. New Phytologist 157, 213-228.

Anfossi, D., Sandroni, S., Viarengo, S., 1991. Tropospheric ozone in the nineteenth century: the Moncalieri series. J Geophys Res 96 (D9), 17349–17352.

Ashmore, M.R., 2005. Assessing the future global impacts of ozone on vegetation. Plant, Cell and Environment 28, 949-964.

Barnes, J.D., Velissariou, D., Davison, A.W., Holevas, C.D., 1990. Comparative ozone sensitivity of old and modern Greek cultivars of spring wheat. New Phytologist, 116, 707-714.

Bassin, S., Volk, M., Suter, M., Buchmann, N., Fuhrer, J., 2007. Nitrogen but not ozone affects productivity and species composition of subalpine grassland after 3 yr of treatment. New Phytol 175, 523–534.

Black, V.J., Black, C.R., Roberts, J.A., Stewart, C.A., 2000. Impact of ozone on the reproductive development of plants. New Phytologist 147, 421-447.

Bergmann, E., Bender, J., Weigel, H.J., 2015. Assessment of the impacts of ozone on biodiversity in terrestrial ecosystems: Literature review and analysis of methods and uncertainties in current risk assessment approaches Part II: Literature review of the current state of knowledge on the impact of ozone on biodiversity in terrestrial ecosystems. Thünen-Institut für Biodiversität, Braunschweig. Federal Environment Agency (Germany).

Bermejo, V., 2002. Efectos del ozono sobre la producción y la calidad de frutos de Lycopersicon esculentum. Modulación por diversos factores ambientales. Tesis doctoral. Universidad Autónoma de Madrid.

Bobbink, R., Hicks, K., Galloway, J., Spranger, T., Alkemade, R., Ashmore, M., Bustamante, M., Cinderby, S., Davidson, E., Dentener, F., Emmett, B., Erisman, J-W., Fenn, M., Gilliam, F., Nordin, A., Pardo, L., de Vries, W., 2010. Global assessment of nitrogen deposition effects on plant terrestrial biodiversity: a synthesis. Ecological Applications 20, 30-59.

Bonanomi, G., Caporaso S., Allegrezza, M., 2009. Effects of nitrogen enrichment, plant litter removal and cutting on a species-rich Mediterranean calcareous grassland. Plant Biosystems 143, 443-455.

Bouvier-Brown, N.C., Goldstein, A.H., Gilman. J.B., Kuster. W.C., de Gouw. J.A, 2009. In situ ambient quantification of monoterpenes, sesquiterpenes, and related oxygenated compounds during BEARPEX 2007: implications for gas and particle-phase chemistry. Atmospheric Chemistry and Physics Discussion 9, 10235–10269.

Calvete-Sogo, H., Elvira, S., Sanz, J., González-Fernández, I., García-Gómez, H., Sánchez-Martín, L., Alonso, R., Bermejo, V., 2014. Current ozone levels threaten gross primary production and yield of Mediterranean annual pastures and nitrogen modulates the response. Atmospheric Environment 95, 197–206.

Calvete-Sogo H., González-Fernández I., Sanz J., Elvira S., Alonso R., García-Gómez H., Ibáñez-Ruiz MA., Bermejo-Bermejo V. 2016. Heterogeneous responses of component species to ozone and nitrogen alter the species composition of Mediterranean annual pastures. Oecologia, http://dx.doi.org/10.1007/s00442-016-3628-z.

Cape, J.N., 2008. Surface ozone concentrations and ecosystem health: Past trends and a guide to future projections. Science of the Total Environment 400, 257-269.

CLRTAP, 2010. Mapping Critical Levels for Vegetation, Chapter III of Manual on methodologies and criteria for modelling and mapping critical loads and levels and air pollution effects, risks and trends. UNECE Convention on Long-range Transboundary Air Pollution; accessed [date of consultation] on Web at www.icpmapping.org .

Chaudhary, N., Agrawal, S. B., 2013. Intraspecific responses of six Indian clover cultivars under ambient and elevated levels of ozone. Environmental Science and Pollution Research 20, 5318-5329.

Chameides, W. & Walker, J., 1973. A photochemical theory of tropospheric ozone. Oceans and Atmospheres 78, 8751-8760.

Chipperfield, M. P., Dhomse, S.S., Feng, W., McKenzie, R.L., Velders, G.J.M., Pyle, J. A., 2015. Quantifying the ozone and ultraviolet benefits already achieved by the Montreal Protocol. Nature Communications 6, Article number: 7233 doi:10.1038/ncomms8233.

Cristofanelli, P., Scheel, H. E., Steinbacher, M., Saliba, M., Azzopardi, F., Ellul, R., ... & Bonasoni, P., 2015. Long-term surface ozone variability at Mt. Cimone WMO/GAW global station (2165 m asl, Italy). Atmospheric Environment 101, 23-33.

Crutzen, P.J., 1974. Photochemical reactions initiated by and influencing ozone in unpolluted tropospheric air. Tellus 26, 47-57.

Danielsson, H., Karlsson, G.P., Karlsson, P.E., Pleijel, H., 2003. Ozone uptake modelling and flux response relationships an assessment of ozone-induced yield loss in spring wheat. Atmospheric Environment 37, 475-485.

Darrall, N.M. 1989. The effect of air pollutants on physiological processes in plants. Plant Cell and Environment 12, 1-30.

Dawnay, L., Mills, G., 2009. Relative effects of elevated background ozone concentrations and peak episodes on senescence and above-ground growth in four populations of Anthoxanthum odoratum L. Environmental Pollution 157, 503-510.

Dias, T., Malveiro, S., Martins-Loução, M.A., Sheppard, L., Cruz, C., 2011. Linking increased N-driven biodiversity changes with soil N availability in a Mediterranean-Type Ecosystem. Plant and Soil 341, 125–136.

Dizengremel, P., Le Thiec, D., Bagard, M., Jolivet, Y, 2008. Ozone risk assessment for plants: central role of metabolism-dependent changes in reducing power. Environmental Pollution 156, 11–15.

Dueñas, C., Fernández, M.C., Cañete, S., Carretero, J., Liger, E., 2004. Analyses of ozone in urban and rural sites in Malaga (Spain). Chemosphere 56, 631–639.

Duprè, C., Stevens, C. J., Ranke, T., Bleeker, A., Peppler-Lisbach, C., Gowing, D. J. G., Dise, N. B., Dorland, E., Bobbink, R., and Diekmann, M., 2010. Changes in species richness and composition in European acidic grasslands over the past 70 years: the contribution of cumulative atmospheric nitrogen deposition. Global Change Biology 16, 344-357.

Del Rio, L.A., Sandalio, L.M., Corpas, F.J., Palma, J.M., Barroso, J.B., 2006. Reactive oxygen species and reactive nitrogen species in peroxisomes. Production, scavenging, and role in cell signaling. Plant Physiology, 141, 330–335.

Díaz, M. & Pulido, F. J., 2009. 6310 Dehesas perennifolias de Quercus spp. En: VV.AA., Bases ecológicas preliminares para la conservación de los tipos de hábitat de interés comunitario en España. Madrid: Ministerio de Medio Ambiente, y Medio Rural y Marino.

Elvira, S., Alonso, R., Castillo, F.J., Gimeno, B.S., 1998. On the response of pigments and antioxidants of Pinus halepensis seedlings to Mediterranean climatic factors and long-term ozone exposure. New Phytologist 138, 419-432.

Elvira, S., Alonso,R., González-Fernández, I., Sanz, J., Bermejo, V. Elvira et al., 2016 Ozone levels in the Spanish Sierra de Guadarrama mountain range are above the thresholds for plant protection: analysis at 2262, 1850 and 995 m a.s.l. Environmental Monitoring and Assessment. DOI 10.1007/s10661-016-5581-z

ENA (European Nitrogen Assessment), 2011. Sutton M.A., Howard C.M., Erisman J.W. et al. (Eds.). The European Nitrogen Assessment. Sources, effects and policy perspectives. Cambridge University Press, Cambridge.

Emberson, L.D., Ashmore, M.R., Cambridge, H.M., Simpson, D., Tuovinen, J.P., 2000. Modelling stomatal ozone flux across Europe. Environmental Pollution 109, 403-413.

Emberson, L., Simpson, D., Tuovinen, J.P., Ashmore, M., Cambridge, H., 2000. Towards a model of ozone deposition and stomatal uptake over Europe. EMEP MSC-W Note 6/2000. The Norwegian Meteorological Institute, Oslo, Norway.

Fares, S., Goldstein, A., Loreto, F., 2010. Determinants of ozone fluxes and metrics for ozone risk assessment in plants. Journal of Experimental Botany 61, 629-633.

Farré Armengol, G., Peñuelas, J., Yli Pirilä, T. Li, P., Filella, I., Llusia, J., 2016. Ozone degrades floral scent and reduces pollinator attraction to flowers. New Phytologist 209 (1), 152-160.

Feng, Z., Kobayashi, K., Ainsworth, E.A., 2008. Impact of elevated ozone concentration on growth, physiology, and yield of wheat (Triticum aestivum L.): a meta-analysis. Global Change Biology 14, 2696-2708.

Fenn, M.E., Poth, M., Bytnerowicz, A., Sickman, J.O., Takemoto, B.K., 2003. Effects of ozone, nitrogen deposition and other stressors on montane ecosystmes in the Sierra Nevada.In: Bytnerowicz, A., Arbaugh, M., Alonso, R. (eds.), Ozone air pollution in the Sierra Nevada: distribution an effects on forests. Elsevier, UK, pp. 111-157.

Fiscus, E.L., Booker, F.L., Burkey, K.O., 2005. Crop responses to ozone: uptake, modes of action, carbon assimilation and partitioning. Plant, Cell and Environment 28, 997-1011.

Fernandez-Fernandez, M.I., Gallego, M.C., Garcia, J.A., Acero, F.J., 2011. A study of surface ozone variability over the Iberian Peninsula during the last fifty years. Atmospheric Environment 45, 1946-1959.

Foot, J.P., Caporn, S.J.M., Lee, J.A., Ashenden, T.W., 1996. The effect of long term ozone fumigation on the growth, physiology and frost sensitivity of Calluna vulgaris. New Phytologist 133, 503-511.

Fuhrer, J., Skärby, L., Ashmore, M.R., 1997. Critical level for ozone effects on vegetation in Europe. Environmental Pollution 97, 91-106.

Fuhrer, J., Booker, F., 2003. Ecological issues related to ozone: agricultural issues. Environment International 29, 141-154.

Gaastra, P., 1959. Photosynthesis of crop plants as influenced by light, carbon dioxide, temperature and stomatal diffusion resistance. Mededelingen van de Landbouwhoogeschool te Wageningen 59, 1-68.

Gallardo, A., Covelo, F., Morillas, L., Delgado, M., 2009. Nutrient cycling and soil processes in terrestial ecosystems: specific nature of mediterranean ecosystem and its implications for the soil-plant relationship. Ecosistemas 18, 4-19.

Garcia, J.M., Alonso, R., Benavides, R., Roig, S., 2014. Species richness of vascular plants along the climatic range of the Spanish dehesas at two spatial scales. Forest Systems 23 (1), 111-119.

García, M.A., Sánchez, M.L., Pérez, I.A., De la Torre, B. 2005 Ground level ozone concentrations at a rural location in northern Spain. Sci Total Environ 348, 135–150.

Garcia-Gomez, H., Garrido, J.L., Vivanco, M.G., Lassaletta, L., Rabago, I., Avila, A., Tsyro, S., Sanchez, G., Gonzalez-Ortiz, A., Gonzalez-Fernandez, I., Alonso, R., 2014 Nitrogen deposition in Spain: modeled patterns and threatened habitats within the Natura 2000 network. Sci Total Environ 485:450–460.

Gauss, M., Myhre, G., Isaksen, I. S. A., Grewe, V., Pitari, G., Wild, O., Collins, W. J., Dentener, F. J., Ellingsen, K., Gohar, L. K., Hauglustaine, D. A., Iachetti, D., Lamarque, J. F., Mancini, E.,Mickley, L. J., Prather, M. J., Pyle, J. A., Sanderson, M. G., Shine, K. P., Stevenson, D. S., Sudo, K., Szopa, S., Zeng, G., 2006. Radiative forcing since preindustrial times due to ozone change in the troposphere and the lower stratosphere. Atmospheric Chemistry and Physics 6, 575-599.

Gilliland, N.J., Chappelka, A.H., Muntifering R.B., Fitzgerald L.B., 2012. Digestive utilization of ozone-exposed forage by rabbits (Oryctolagus cuniculus). Environmental Pollution 163, 281-286.

Gimeno, B.S., Bermejo, V., Reinert, R., Zheng, Y., Barnes, J., 1999. Adverse effects of ambient ozone on watermelon yield and physiology at a rural site in Eastern Spain. New phytologist 144, 245-260.

González-Fernández, I., Bermejo, V., Alonso, R., Elvira, S., Sanz, J., Gimeno, B.S., 2010. Modelling dehesa annual pasture dynamics: application to stomatal ozone deposition. Atmospheric Environment 44, 2507-2517.

González-Fernandez, I., Calvo, E., Gerosa, G., Bermejo, V., Marzuoli, R., Calatayud, V., Alonso, R., 2014. Setting ozone critical levels for protecting horticultural Mediterranean crops: case study of tomato. Environmental Pollution 185, 178-187.

Gruber, N.& Galloway, J., 2008. An Earth-system perspective of the global nitrogen cycle. Nature 451, 293-296.

Grulke, N., Minnich, R., Paine, T., Seybold, S., Chavez, D., Fenn, M., Riggan, P., Dunn, A., 2008. Chapter 17 Air Pollution Increases Forest Susceptibility to

Wildfires: A Case Study in the San Bernardino Mountains in Southern California. Developments in Environmental Science Volume 8, 365-403.

Grulke, N.E., Andersen, C.P., Fenn, M.E., Miller, P.R., 1998. Ozone exposure and nitrogen deposition lowers root biomass of ponderosa pine in the San Bernardino Mountains, California. Environmental Pollution 103, 63-73.

Grulke, N.E., Minnich, R.A., Paine, T.D., Seybold, S.J., Chavez, D.J., Fenn, M.E.,Riggan, P.J., Dunn, A., 2009. Air pollution increases forest susceptibility towildfires: a case study in the San Bernardino Mountains in southern California.In: Bytnerowicz, A., Arbaugh, M.J., Riebau, A.R., Andersen, C. (Eds.), WildlandFires and Air Pollution. Developments in Environmental Science, vol. 8. Elsevier,Amsterdam, pp. 365-403.

Grünhage, L., Krupa, S.V., Legge, A.H., Jäger, H.J., 2004. Ambient flux-based critical values of ozone for protecting vegetation: differing spatial scales and uncertainties in risk assessment. Atmospheric Environment 38, 2433-2437.

Guicherit, R., Roemer, M., 2000. Troposheric ozone trends. Chemosphere-Global Change Science 2, 167-183.

Halliwell, B., 2006. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiology 141, 312-322.

Heagle, A.S., Body, D.E., Heck, W.W., 1973. An open top chamber to assess the impact of air pollution on plants. J. Environ. Quality. Vol 2 (3): 365-368.

Heagle, A. S., Philbeck, R. B., Rrogers, H. H., Letchworth, M. B., 1979. Dispensing and monitoring ozone in open-top chambers for plant-effects studies. Phytopathology, 69(1): 15-20.

Heath, R.L., 2008. Modification of the biochemical pathways of plants induced by ozone: what are the varied routes to change? Atmospheric Environment 155, 453-463.

Heggestad, H. E., Middleton, J. T., 1959. Ozone in high concentrations as a cause of tobacco leaf injury. Science 129, 208-210.

Hewitt, D.K.L., Mills, G., Hayes, F., Wilkinson, S., Davies, W., 2014. Highlighting the threat from current and near-future ozone pollution to clover in pasture Environmental Pollution 189, 111-117.

Jarvis, P.G. 1976. The interpretation of the variations in leaf water potential and stomatal conductance found in canopies in the field. Philosophical Transactions of the Royal Society, London B 273, 593-610.

Jones, M.E., Paine, T.D., Fenn, M.E., Poth, M.A., 2004. Influence of ozone and nitrogen deposition on bark beetle activity under drought conditions. Fisheries Management and Ecology 200, 67-76.

Kangasjarvi, J., Talvinen, J., Utrianien, M., Karjalainen, R., 1994. Plant defense systems induced by ozone. Plant Cell Environ, 17, 783-794.

Kärenlampi L, Skärby L. (Eds.), 1996. Critical Levels for Ozone in Europe: Testing and Finalising the Concepts. UNECE Workshop report, Kuopio, Finland.

Karlsson PE, Sellden G, Pleijel H. (Eds.), 2003. Establishing Ozone Critical Levels II.UN/ECE Workshop Report. IVL Report B 1523, Swedish Environmental Research Institute, Gothenburg. Available at: http://www.ozoneworkshop.ivl.se/.

Laisk, A., Kull, O., Moldau, H., 1989. Ozone concentration in leaf intercellular air spaces is close to zero. Plant Physiology 90, 1163-1167.

Lindroth, R.L., B.J. Kopper, W.F.J. Parsons, J.G. Bockheim, D.F. Karnosky, G.R. Hendrey, K.S. Pregitzer, J. Isebrands, and J. Sober. 2001. Consequences of elevated carbon dioxide and ozone for foliar chemical composition and dynamics in trembling aspen (*Populus tremuloides*) and paper birch (Betula papyrifera). Environmental Pollution 115, 395-404.

Marañon, T., 1985. Diversidad florística y heterogeneidad ambiental en una dehesa de Sierra Morena. Anales de Edafología y Agrobiología 44, 1183-1197.

Marenco, A., Gouget, H., Nédélec, P., Pagés, J.P., Karcher, F. 1994. Evidence of a long-term increase in tropospheric ozone from Pic du Midi data series consequences positive radiative forcing. Journal of Geophysical Research-Atmospheres 99, 16617-16632.

Mc Laughlin, S.B., McConathy, R.K., 1983. Effects of SO_2 and O_3 on allocation of 14C labeled photosynthate in Phaseolus vulgaris. Plant Physiology 73, 630–635.

Mc Laughlin, S.B., Nosal, M., Wullschleger, S.D., Sun, G., 2007. Interactive effects of ozone and climate on water use, soil moisture content, and streamflow in a Southern Appalachian Forest in the USA. New Phytologist 174, 125e136.

Massman, W.J., 2004. Towards an ozone standard to protect vegetation based on effective dose: a review of deposition resistances and a possible metric. Atmospheric Environment 38, 2323-2337.

Millán, M., Salvador, R., Mantilla, E., Kallos, G., 1997. Photooxidant dynamics in the Mediterranean basin in summer: Results from European Research projects. J Geophys Res 102(D7):8881–8823.

Millán, M., Sanz, M.J., Salvador, R., Mantilla, E., 2002. Atmospheric dynamics and ozone cycles related to nitrogen deposition in the western Mediterranean. Environmental Pollution 118, 167-186.

Mills, G., Hayes, F., Simpson, D., Emberson, L., Norris, D., Harmens, H. and Buker, P., 2011. Evidence of widespread effects of ozone on crops and (semi-)natural vegetation in Europe (1990–2006) in relation to AOT40- and flux-based risk maps. Global Change Biology 17, 592–613.

Misselbrook T., Del Prado A., Chadwick D., 2013. Opportunities for reducing environmental emissions from forage-based dairy farms. Agriculture and Food Science. 22, 93–107.

Monks, P. S., Granier, C., Fuzzi, S., Stohl, A., Williams, M. L., Akimoto, H., ... & Prevot, A. S. H., 2009. Atmospheric composition change–global and regional air quality. Atmospheric Environment 43(33), 5268-5350.

Myers, N., Mittermeier, R.A., Mittermeier, C.,Da Fonseca, G., Kent, J., 2010. Biodiversity hotspots for conservation priorities. Nature 403, 853-858

Navrot, N., Rouhier, N., Gelhaye, E., Jaquot, J.P., 2007. Reactive oxygen species generation and antioxidant systems in plant mitochondria. Physiologia Plantarum, 129, 185–195.

Notario, A., Díaz-de-Mera, Y., Aranda, A., Adame, J.A., Parra, A., Romero, E., Parra, J., Muñoz, F., 2012. Surface ozone comparison conducted in two rural areas in central-southern Spain. Environ. Sci. Pollut. Res. 19, 186–200.

Notario, A., Bravo, I., Adame, J.A., Díaz-de-Mera, Y., Aranda, A., Rodríguez, A., Rodríguez, D., 2013. Variability of oxidants ($OX = O_3 + NO_2$), and preliminary study on ambient levels of ultrafine particles and VOCs, in an important ecological area in Spain. Atmospheric Research 128, 35-45.

Olea, L., San Miguel-Ayanz, A., 2006. The Spanish dehesa. A traditional Mediterranean silvopastoral system linking production and nature conservation. Grassland Science in Europe 11, 1-15.

Oltmans, S., Lefohn, A., Harris, J., Galbally, I., Scheel, H., Bodeker G., et al., 2006. Long term changes in tropospheric ozone. Atmospheric Environment 40, 3156–3173.

Pell, E.J., Schlagnhaufer, C.D., Arteca, R.N., 1997. Ozone-induced oxidative stress: Mechanisms of action and reaction. Physiologia Plantarum 100, 264-273.

Peco, B., Rico, L., Azcárate F.M., 2009. Seed size and response to rainfall patterns in annual grasslands: 16 years of permanent plots. Journal of Vegetation Science 20, 8-16.

Peñuelas, J., Ribas Carbó, M., González Meler, M., Azcón Bieto, J., 1994. Water status, photosynthetic pigments, C/N ratios and respiration rates of sitka spruce seedlings exposed to 70 ppbv ozone for a summer. Environmental and Experimental Botany 34(4), 443-449.

Pryor, M.V., Church, D.F., 1991. Aldehydes, hydrogen peroxide, and organic radicals as mediators of ozone toxicity. Free Radic. Biol. Med. 11, 41-46.

Plaza, J., Pujadas, M., Artíñano, B., 1997. Formation and transport of the Madrid ozone plume. Journal of Air & Waste Management Association 47, 666-774.

Pleijel, H., Danielsson, H., Gelang, J., Sild, E., Selldén, G., 1998. Growth stage dependence of the grain yield response to ozone in spring wheat (Triticum aestivum L.). Agriculture, Ecosystems and Environment 70, 61-68.

Pleijel, H., Danielsson, H., Emberson, L., Ashmore, M.R., Mills, G., 2007. Ozone risk assessment for agricultural crops in Europe: Further development of stomatal flux and flux-response relationships for European wheat and potato. Atmospheric Environment 41, 3022-3040.

Pujadas, M., Terés, J., Gimeno, B.S., 1997. La experiencia española en el diseño de sistemas experimentales para el estudio de los efectos producidos por los contaminantes gaseosos sobre especies vegetales. Boletín de Sanidad Vegetal. Plagas, 23, 39-55.

Richards, B.L., Middleton, J.T., Hewitt, W.B., 1958. Air pollution with relation to agronomic crops. V. Oxidant stipple of grape. Agron. J. 50, 559-561.

Rivas Goday, S., Rivas Martinez, S., 1963. Estudio y clasificación de los pastizales españoles. Ministerio de Agricultura. Madrid.

Ribas, A., Peñuelas, J., 2003. Biomonitoring of tropospheric ozone phytotoxicity in rural Catalonia. Atmospheric Environment 37, 63-71.

Rodà, F., Ávila, A., Rodrigo, A., 2002. Nitrogen deposition in Mediterranean forests. Environmental Pollution 118, 205-213.

Roig, S., Evett, R., Gea-Izquierdo, G., Cañellas, I., Sánchez-Palomares, O., 2013. Climatic Influence on Oak Landscape Distributions. Mediterranean Oak Woodland Working Landscapes. Volume 16 of the series Landscape Series pp 61-89. Date:19 June 2013. ISBN: 978-94-007-6706-5.

Sala, O.E., Chapin III, F.J., Armesto, J.J., Berlow, E., Bloomfield, J., Dirzo, R., Huber-Sanwald, E., Huenneke, L., Jackson, R.B., Kinzig, A., Leemans, R., Lodge, D., Mooney, H., Oesterheld, M., Poff, N.L., Sykes, M.T., Walker, B.H., Walker, M., Wall, D.H., 2000. Global diversity scenarios for the year 2100. Science 287, 1770–1774.

Sanz, M. J., Carratalá, A., Gimeno, C., and Millán, M. M., 2002. Atmospheric nitrogen deposition on the east coast of Spain: relevance of dry deposition in semi-arid Mediterranean regions. Environmental Pollution 118, 259-272.

Schraudner, M., Langebartels, C., Sandermann, H., 1997. Changes in the biochemical status of plant cells induced by the environmental pollutant ozone. Physiologia Plantarum 100, 274-280.

Skärby, L., Ro-Poulsen, H., Wellburn, F.A.M., Sheppard, L.J., 1998. Impacts of ozone on forests: a European perspective. New Phytologist 139, 109-122.

Sánchez, D., Elena Rosselló, R., Roig, S., García del Barrio, J.M., 2007. Los paisajes de dehesa en España y su relación con el ambiente geoclimático. Cuadernos de la Sociedad Española de Ciencias Forestales. Actas de la III reunión sobre sistemas forestales. Nº. 22, 171-175.

Sandroni, S., Anfossi, D., Viarengo, S., 1992. Surface ozone levels at the end of the nineteenth century in South America. Journal of Geophysical Research, 97, 2535-2539.

San Miguel Ayanz, A., 1998. La dehesa Española. Fundación Conde del Valle de Salazar. Madrid.

San Miguel Ayanz, A., 2001. Pastos naturales españoles. Editorial Mundi Prensa. Madrid.

Santamaria, O., García White, T., 2010. Dinámica productiva de forrajes de diferentes cultivares de triticales y avenas como suplemento de los pastos de dehesa y pastizales del S.O: de la Península Ibérica. Pastos: Fuente Natural de Energia. Universidad de León.

Santamaria, O.; Poblaciones, M.J.; Olea, L.; Rodrigo, S.; Viguera, F.J.; Garcia White, T., 2009. Influencia de nuevos fertilizantes sobre la producción de biomasa y parámetros de calidad en pastos de dehesa del SO de España. En: La multifuncionalidad de los pastos: producción ganadera sostenible y gestión de los ecosistemas.

Singh Gill, S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry 48, 909-930.

Simpson, D., Fagerli, H., Jonson, J., Tsyro, S., Wind, P., Tuovinen, J.P., 2003. The EMEP Unified Eulerian Model. Model Description. EMEP MSC-W Report 1/2003. The Norwegian Meteorological Institute, Oslo, Norway.

Simpson, D., Emberson, L., Ashmore, M., Tuovinen, J., 2007. A comparison of two different approaches for mapping potential ozone damage to vegetation. A model study. Environmental Pollution 146, 715-725.

Sitch, S., Cox, P.M., Collins, W.J., Huntingford, C., 2007. Indirect radiative forcing of climate change through ozone effects on the land-carbon sink. Nature 448, 791-794.

Schmidt, U., Thöni, H., Kaupenjohann, M., 2000. Using a boundary line approach to analyze N_2O flux data from agricultural soils. Nutrient Cycling in Agroecosystems 57, 119-129.

Snyder, C.S., T.W. Bruulsema, T.L. Jensen, and P.E. Fixen. 2009. Review of greenhouse gas emissions from crop production systems and fertilizer management effects. Agriculture, Ecosystems and Environment 133:247–266.

Stampfli, A., Fuhrer, J., 2010. Spatial heterogeneity confounded ozone-exposure experiment in semi-natural grassland. Oecologia 162 (2), 515-522.

Stevens, C.J., Dise, N.B., Mountford, J.O., Gowing, D.J., 2004. Impact of nitrogen deposition on the species richness of grasslands. Science 303, 1876-1879.

Stevens, C.J., Dise, N.B., Gowing, D.J., Mountford, J.O., 2006. Loss of forb diversity in relation to nitrogen deposition in the UK: regional trends and potential controls. Global Change Biology 12, 1823-1833.

Sutton, M. A., Howard, C., Erisman, J. W., Billen, G., Bleeker, A., Grennfelt, P., van Grinsven, H., and Grizzetti, B.: The European Nitrogen Assessment, Cambridge University Press, 612 pp., 2011.

Takemoto, B.K., Bytnerowicz, A., Fenn, M.E., 2001. Current and future effects of ozone and atmospheric nitrogen deposition on California's mixed conifer forests. Forest Ecology and Management 144, 159-173.

Uddling, J.,Karlsson, P.E.,Glorvigen, A.,Selldén, G., 2006. Ozone impairs autumnal resorption of nitrogen from birch (Betula pendula) leaves, causing an increase in whole-tree nitrogen loss through litter fall. Tree Physiol., 26(1),113-20.

Van Zelm, R., Huijbregts, M., Den Hollander, H., Van Jaarsveld, H., Sauter, F., Struijs, J., Van Wijnen, H., Van de Meent, D., 2008. European characterization factors for human health damage of PM10 and ozone in life cycle impact assessment. Atmospheric Environment 42, 441-453.

Vazquez-de-Aldana, B.R., Garcia-Ciudad., A., Garcia-Criado., 2008. Interannual variations of aboveground biomass and nutritional quality of Mediterranean grasslands in Western Spain over a 20-year period. Australian Journal of Agricultural Research 59, 769-779.

Villanueva, F., Tapia, A., Notario, A., Albaladejo, J., Martínez E., 2014. Ambient levels and temporal trends of VOCs, including carbonyl compounds, and ozone at Cabañeros National Park border, Spain. Atmospheric Environment 85, 256-265.

Vingarzan, R., 2004. A review of surface ozone background levels and trends. Atmospheric Environment 38, 3431-3442.

Volk, M., Bungener, P., Contat, F., Montami, M., Fuhrer, J., 2006. Grassland declined by a quarter in 5 years of free-air ozone fumigation. Global Change biology 12, 74-83.

Vollenweider, P., Günthardt-Goerg, M.S., 2005. Diagnosis of abiotic and biotic stress factors using the visible symptoms in foliage. Environ. Pollut. 137, 455-465.

Wieser, G., Matyssek, R., 2007. Linking ozone uptake and defense towards mechanistic risk assessment for forest trees. New Phytologist 174, 7-9.

Witting, V.E., Ainsworth. E.A., Naidu. S.L., Karnosky. D.F., Long. S.P., 2009. Quantifying the impact of 914 current and future tropospheric ozone on tree biomass, growth, physiology and biochemistry: a 915 quantitative meta-analysis. Global Change Biology, 15, 396-424.

Vollsnes, A.V., Kruse, O.M.O., Eriksen, A.B., Oxaal, U., Futsaether, C.M., 2010. In vivo root growth dynamics of ozone exposed Trifolium subterraneum. Environmental and Experimental Botany 69, 183-188.

Wedlich, K., Rintoul, N., Peacock, S., Cape, J.N., Coyle, M., Toet, S., Barnes, J., Ashmore, M., 2012. Effects of ozone on species composition in an upland grassland. Oecologia, 168, 1137–1146.

Weinstein, D.A., R.M. Beloin and R.D. Yanai. 1991. Modeling changes in red spruce carbon balance and allocation in response to interacting ozone and nutrient stress. Tree Physiol. 9:127–146.

Wyness, K., Mills, G., Jones, L., Barnes, J., Jones D., 2011. Enhanced nitrogen deposition exacerbates the negative effect of increasing background ozone in Dactylis glomerata, but not Ranunculus acris. Environmental Pollution 159, 2493-2499.

Van Zelm, R., Huijbregts, M., Den Hollander, H., Van Jaarsveld, H., Sauter, F., Struijs, J., Van Wijnen, H., Van de Meent, D., 2008. European characterization factors for human health damage of PM10 and ozone in life cycle impact assessment. Atmospheric Environment 42, 441-453.

2. OBJETIVOS

Los objetivos de la presente memoria se dividen en cuatro apartados:

1. Análisis de la respuesta al incremento del O_3 troposférico en especies pascícolas mediterráneas anuales características de pastizales de dehesa, representativas de dos familias de gran interés ecológico y pastoral: *Leguminosae* y *Gramineae*, mediante los siguientes parámetros:

- Análisis de daños visibles en hojas
- Análisis de parámetros de biomasa
- Análisis de la fenología
- Análisis de parámetros de capacidad reproductiva
- Análisis de parámetros e índices de calidad nutritiva

2. Análisis de la interacción entre el incremento de O_3 troposférico y el incremento de la disponibilidad de N simulando el depósito atmosférico. Se consideran las mismas especies y parámetros del punto anterior.

3. Cuantificación general del efecto del O_3 en especies pascícolas anuales mediante funciones de exposición y dosis-respuesta para los parámetros estudiados. Análisis de los diferentes índices y selección de los más adecuados.

4. Revisión y propuesta de los niveles críticos de O_3 para vegetación herbácea (seminatural) dominada por especies anuales en el marco del CLRTAP/UNECE.

Para alcanzar los objetivos propuestos se desarrollaron tres fases experimentales y un análisis conjunto de toda la información disponible:

La primera fase experimental analiza el rango de respuesta al incremento de O_3 troposférico de diferentes especies pascícolas anuales mediterráneas. Se desarrolla en el apartado 3.1. (Análisis de sensibilidad al ozono de especies pascícolas mediterráneas) y se corresponde con dos publicaciones SCI:

- Bermejo, V., Gimeno, B.S., Sanz, J., de la Torre, D., Gil, J.M., 2003. Assessment of the ozone sensitivity of 22 native plant species from Mediterranean annual pastures based on visible injury. Atmospheric Environment 37,4667-4677.

- Gimeno, B.S., Bermejo, V., Sanz, J., de la Torre, D., Elvira, S., 2004. Growth response to ozone of annual species from Mediterranean pastures. Environmental Pollution 132, 297-306.

La segunda fase experimental analiza el efecto del O_3 en la capacidad reproductiva de especies las herbáceas anuales. Se desarrolla en el apartado 3.2 (Efectos del ozono en la capacidad reproductiva de tres tréboles mediterráneos considerando el factor competencia) y se corresponde con una publicación SCI:

- Gimeno BS., Bermejo V., Sanz, J., de la Torre, D. and Gil, J.M., 2004. Assessment of the effects of ozone exposure and plant competition on the reproductive ability of three therophytic clover species from Iberian pastures. Atmospheric Environment 38, 2295-2303.

La tercera fase experimental se ha desarrollado para analizar la modulación de la respuesta al O_3 de las especies anuales debido al incremento de la disponibilidad de N simulando el depósito atmosférico. Se desarrolla en el capítulo 3.3 (Modulación de la respuesta al ozono de especies pascícolas mediterráneas anuales por la disponibilidad de nitrógeno) y se corresponde con cinco publicaciones SCI:

- Sanz, J., Muntifering, R.B., Bermejo, V., Gimeno, B.S., Elvira, S., 2005. Ozone and increased nitrogen supply effects on the yield and nutritive quality of *Trifolium subterraneum*. Atmospheric Environment 39, 5899-5907.
- Sanz, J., Bermejo, V., Gimeno, B., Elvira, S., Alonso, R., 2007. Ozone sensitivity of the Mediterranean terophyte *Trifolium striatum* is modulated by soil nitrogen content. Atmospheric Environment 41, 8952-8962.
- Sanz, J., Bermejo, V., Muntifering, R.B., Gonzalez-Fernandez, I., Gimeno. B., Elvira, S., Alonso, R., 2011. Plant phenology, growth and nutritive quality of *Briza maxima*: Responses induced by enhanced ozone atmospheric levels and nitrogen enrichment. Environmental Pollution 159, 423-430.
- Sanz J, Calvete-Sogo H, González-Fernández I, Lin J, García-Gómez H, Muntifering R, Alonso R, Bermejo-Bermejo V., 2013. Foliar senescence is the most sensitive response to ozone in *Bromus hordeaceus* and is modulated by nitrogen input. Grass and Forage Science. doi: 10.1111/gfs.12090.

- J. Sanz, I. González-Fernández, H. Calvete-Sogo, J.S. Lin, R. Alonso, R. Muntifering, V. Bermejo., 2014. Ozone and nitrogen effects on yield and nutritive quality of the annual legume *Trifolium cherleri*. Atmospheric Environment, 94, 765-772.

La fase final tiene como objetivo general el análisis conjunto de las bases de datos obtenidas en la fase experimental para la cuantificación de la respuesta general al O_3, la construcción de funciones de respuesta al contaminante y la revisión y definición de niveles críticos de O_3 para las comunidades dominadas por especies anuales en el marco del CLRTAP/UNECE. Se desarrolla en el apartado 3.4. (Propuesta de niveles críticos para ozono en especies pascícolas mediterráneas) y se corresponde con una publicación SCI:

- Sanz, J., González-Fernández, I., Elvira, S., Muntifering, R., Alonso, R., Bermejo-Bermejo, V., 2016. Setting ozone critical levels for annual Mediterranean pasture species: combined analysis of Open-Top chamber experiments. Science of the Total Environment, 571, 670-679.

3. RESULTADOS

3.1. ANÁLISIS DE SENSIBILIDAD AL OZONO DE ESPECIES PASCÍCOLAS MEDITERRÁNEAS ANUALES

Se presentan los resultados correspondientes a la valoración de los efectos del O_3 en parámetros de crecimiento y daños foliares en 22 terófitos representativos de comunidades pascícolas de la dehesa. Los experimentos se desarrollaron en una instalación de cámaras de techo descubierto (OTCs). Se consideraron tres tratamientos de O_3: aire filtrado, cuya concentración no sobrepasa las 15 ppb (CFA); aire no filtrado, que reproduce las concentraciones ambientales del contaminante (NFA); y aire no filtrado al que se le añaden 40 ppb de O_3 por encima del ambiente (NFA+). Se utilizaron especies de las familias Leguminosae y Gramineae, las dos familias con mayor representación en esas comunidades y con un elevado valor ecológico y pascícola. Las especies pertenecientes a las leguminosas fueron: *Trifolium striatum*, *T. cherleri*, *T. glomeratum*, *T. subterraneum*, *T. hirtum*, *T. angustifolium*, *Biserrula pelecinus*, *Medicago minima*, *Anthyllis lotoides*, *A. cornicina*, *Ornithopus compressus*; y las pertenecientes a las gramíneas: *Avena barbata*, *A. sterilis*, *Aegilops geniculata*, *A. triuncialis, Lolium rigidum*, *Briza maxima*, *Bromus hordeaceus*, *B. sterilis*, *Cynosurus echinatus*, *Micropyrum tenellum*, *Vulpia myurus*.

Figura 10. Especies herbáceas anuales en el interior de una OTC (Autor: J. Sanz).

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Assessment of the ozone sensitivity of 22 native plant species from Mediterranean annual pastures based on visible injury

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Abstract

Ozone (O_3) phytototoxicity has been reported on a wide range of plant species, inducing the appearance of specific foliar injury or increasing leaf senescence. No information regarding the sensitivity of plant species from dehesa Mediterranean grasslands has been provided in spite of their great biological diversity. A screening study was carried out in open-top chambers (OTCs) to assess the O_3-sensitivity of 22 representative therophytes of these ecosystems based on the appearance and extent of foliar injury. A distinction was made between specific O_3 injury and non-specific discolorations. Three O_3 treatments (charcoal-filtered air, non-filtered air and non-filtered air supplemented with $40\,\mathrm{nl\,l^{-1}}$ O_3 during 5 days per week) and three OTCs per treatment were used. The Papilionaceae species were more sensitive to O_3 than the Poaceae species involved in the experiment since ambient levels induced foliar symptoms in 67% and 27%, respectively, of both plant families. An O_3-sensitivity ranking of the species involved in the assessment is provided, which could be useful for bioindication programmes in Mediterranean areas. The assessed *Trifolium* species were particularly sensitive since foliar symptoms were apparent in association with O_3 accumulated exposures well below the current critical level for the prevention of this kind of effect. The exposure indices involving lower cut-off values (i.e. $30\,\mathrm{nl\,l^{-1}}$) were best related with the extent of O_3-induced injury on these species.

Keywords: Grasslands; Dehesa; Clover; Critical levels; Foliar damage

1. Introduction

Tropospheric ozone (O_3) has been reported to reach phytotoxic levels in European rural areas (EMEP, 2002). There is concern about the harm this pollutant might pose to semi-natural grassland vegetation, since it is expected that the different O_3 sensitivity of these plant species might induce changes in the floristic composition of plant communities (Fuhrer et al., 1994). Moreover, grassland ecosystems are particularly endangered by the interaction of other global-change components such as alterations in land-use, climate, nitrogen deposition, biotic exchange or atmospheric carbon dioxide (Sala et al., 2000). Therefore, O_3 effects on these ecosystems should be assessed to effectively reduce anthropogenic impacts on them.

Early experiments concerning O_3 effects on semi-natural plant species were focused on the detection of foliar visible injury following their exposure to elevated O_3 concentrations (Ashmore et al., 1987). Recent research has been carried out exposing herbaceous plants from different communities to realistic O_3 levels. This is the case of the studies carried out by Ashmore et al. (1995), Pleijel and Danielsson (1997), Bergmann et al. (1999) and Franzaring et al. (2000) on the most common herbaceous taxa of northern and central-European flora. However, no information exists on the O_3 sensitivity of herbaceous species from Mediterranean grasslands. This is particularly distressing since plant

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diversity in some of these ecosystems, such as the dehesa-type grasslands, is quite remarkable (Pineda et al., 1981), and also because ambient O_3 levels recorded in the Mediterranean region have been reported as phytotoxic (Fumagalli et al., 2001; Nali et al., 2002; Bermejo et al., 2002). Moreover, recent experimental work (Madkour and Laurence, 2002) has described the high sensitivity of a local Egyptian cultivar of *Trifolium alexandrinum*, driving attention to potential damage in the southern-Mediterranean area.

The dehesas represent an example of sustainable management of natural resources in the Iberian Peninsula, covering agricultural, timber and extensive livestock exploitation. The typical vegetation of dehesa ecosystems is composed of cleared oak woodlands with a grassland understorey. Annual species represents up to 71% of the herbaceous species from these ecosystems (Marañón, 1985). Large spatial and temporal fluctuations in floristic composition occur depending on tree coverage, slope–valley gradients, grazing pressure and meteorological conditions (Peco et al., 1998; San Miguel, 1994). As a result, plant species richness of these ecosystems is quite remarkable, 30 species per 400 cm^2 (Pineda et al., 1981). The most characteristic species of these grasslands belong to the Papilionaceae and Poaceae families. Several studies have reported a great sensitivity to O_3 of Papilionaceae plants in opposition to most of Poaceae species (see the review carried out by Davison and Barnes, 1998). Both taxa have different roles in the ecosystem, mainly in nitrogen cycling, and they also represent dissimilar nutritive sources for the livestock. Therefore, the assessment of the O_3 sensitivity of the plants from these communities is quite appealing since this pollutant might have an impact on the structure, diversity, function and human-utility of these ecosystems belonging to the Mediterranean basin, an area that has been considered as an "hyper-hot" candidate for conservation support (Myers et al., 2000).

When plants are exposed to realistic concentrations, screening studies aiming to assess the sensitivity of a wide range of plants to air pollutants are suitable for the determination of thresholds of injury and may have implications in risk assessment or legislative initiatives. The United Nations Economic Commission for Europe (UN ECE) is leading one of the most comprehensive efforts to control transboundary air pollution through the assessment of the areas, where O_3 levels exceed its phytotoxicity levels, also termed critical levels. *Critical level* has been defined as the pollutant concentration above which direct effects on receptors, such as plants, ecosystems or materials, may occur according to present knowledge (UN ECE, 1988). Ozone critical levels have been proposed for the protection of crops, semi-natural vegetation and forest-tree species (Fuhrer and Achermann, 1999).

The short-term O_3 critical level for the prevention of foliar injury on herbaceous plants was derived from the results of a pan-European experiment carried out under the framework of the International Cooperative Programme (ICP) on the effects of air pollution on natural vegetation and crops (ICP vegetation). This experiment involved the ambient exposure of *T. repens* and *T. subterraneum* following a standardised protocol (Benton et al., 2000). Ozone-induced foliar injury was found to be related to the O_3 exposure corresponding to the 5 days prior to the appearance of visible symptoms. Since air vapour pressure deficit (VPD) modulated O_3 phytotoxicity, the short-term critical level was established at AOT40 (accumulated exposure over a threshold of 40 $nl\,l^{-1}$) values of 200 and 500 $nl\,l^{-1}\,h$ when mean VPD (09:30–16:30) is below or above 1.5 kPa, respectively. Tonneijck and Van Dijk (2002) have reported injury on *T. subterraneum* with AOT40 values below 200 $nl\,l^{-1}\,h$. Therefore, it appears that this critical level needs further revision.

This work presents the results of an experiment that was carried out with the following objectives: (1) to rank O_3 sensitivity of therophytic plants from acidic dehesa pastures according to the appearance of foliar visible injury; (2) to assess whether differences in sensitivity could be attributed to certain plant genus or families, and (3) to evaluate the present short-term critical level for O_3-injury development.

2. Materials and methods

2.1. Experimental site

The experiment was carried out at Sant Jaume d'Enveija, Spain (40°41′N, 0°47′E). No major air pollutant sources that might have affected the results are located in the vicinity of the experimental site.

2.2. Plant material

All the species involved in this study are annual plants from dehesa acidic grasslands (Allué Andrade and Tella, 1986). Annual species are more predominant than perennials due to the extreme meteorological conditions occurring during summer and winter.

Most of the seeds were collected from a typical dehesa located North of Madrid (Dehesa de Moncalvillo, Guadalix de la Sierra, Madrid; 40°40′N 03°46′W). The germoplasm bank of the Agriculture and Environment Council from the Extremadura Autonomous Community supplied the *T. striatum*, *T. subterraneum*, *T. angustifolium* and *Ornithopus compressus* seeds, collected at different sites from central-western areas of the Iberian peninsula. The Spanish *T. subterraneum* cv. Zujar was used in the experiments.

All seeds of Papilionaceae species were immersed during 24 h in a Germinator© solution (Agro-Orgánicos Mediterráneos S.L., Granada, Spain) to ensure homogeneous germination. When seeds were swollen, they were sown in a 50% neutral peat and 50% vermiculite substrate. Poaceae seeds did not experience any pretreatment and they were sown in the same substrate as Papilionaceae seeds. Seedlings were transplanted to 2.5 l pots with a 50% peat, 30% vermiculite and 20% perlite substrate and 2 kg m^{-3} of a slow-release fertiliser (NPK:15/8/11). Plants were irrigated with a droplet system to ensure adequate and homogeneous water availability to plant material.

2.3. Ozone treatments

The same day the plants were transplanted into pots they were introduced in slightly modified NCLAN-type open-top chambers (OTCs) (see Gimeno et al., 1999). Three O_3 treatments were used: charcoal-filtered air (CFA) presenting subphytotoxic O_3 levels, non-filtered air (NFA) with close to ambient O_3 levels and non-filtered air supplemented with 40 nl l^{-1} O_3 from 07:00 to 17:00 (GMT) 5 days week^{-1} (NFA+). Ozone concentrations in the NFA+ treatment were in the range of those reported by Plaza et al. (1997) in the areas where these plant communities are present. Four to six plants of each species were introduced in each chamber and three OTC replicates were used for each O_3 treatment. An automatic system provided a continuous monitoring of O_3, sulphur dioxide and nitrogen oxides concentrations in the different treatments along with meteorological parameters such as wind speed and direction, air temperature and relative humidity, and photosynthetic active radiation (PAR). A complete description of the chambers and the operation of the system is provided in Pujadas et al. (1997) and Alonso et al. (2001). Details on the dates the plants were introduced in the chambers and the length of O_3 exposure in the different treatments can be found in Tables 1 and 2.

2.4. Visible injury assessment

Daily evaluations of O_3-induced visible injury were carried out on plant leaves from the different treatments until the first symptoms were observed in 90% of the plants of a given species. Weekly assessments were performed afterwards. In the last assessment, the number of total leaves, healthy leaves, leaves presenting O_3-specific symptoms and senescent leaves was recorded on three randomly selected stems or tillers per plant on Papilionaceae or Poaceae species, respectively. *T. striatum* and *T. hirtum* were not included in this last assessment. Injury intensity at the plant level was expressed as the percentage of affected to total assessed leaves.

2.5. Ozone exposure indices

The O_3 exposure index AOT40 currently used by both the UN ECE CLRTAP[1] and the European daughter O_3 Directive (2002/3/EC) was calculated for the three O_3 treatments integrating different periods: (a) from the beginning of plant exposure in the chambers until the first visible injury was detected, (b) from the beginning of plant exposure until the last assessment of visible injury was carried out, and (c) integrating the exposure during the 5 days preceding the detection of O_3 injury and determining whether air VPD was below or above 1.5 kPa during this period. The AOT40 index was calculated as the sum of the differences between O_3 hourly concentrations in nl l^{-1} and 40 nl l^{-1} for each hour when concentration exceeds 40 nl l^{-1} and solar radiation is above 50 W m^{-2}.

Ozone exposure throughout the experiment was also calculated using other indices such as AOT30 (accumulated exposure over the cut-off of 30 nl l^{-1}), AOT60 (accumulated exposure over the cut-off of 60 nl l^{-1}) and the number of hours presenting O_3 levels above 30, 40 and 60 nl l^{-1} (NUM30, NUM40 and NUM60, respectively). In addition, O_3 10-h mean (07:00–17:00 GMT), the 24-h O_3 mean, the number of days where the O_3 daily mean exceeded 33 nl l^{-1} (D33), the number of hourly averages exceeding 100 nl l^{-1} (NUM100) and the maximum hourly levels (Max. hourly levels) were also determined.

2.6. Statistical analyses

Ozone effects on visible injury-related parameters were tested performing an ANOVA analysis for each species and variable. In addition, a two-way ANOVA analysis using O_3 treatment and family as factors was carried out to assess whether a given family could be associated with differential O_3 sensitivity. When significant differences ($p<0.05$) were detected, the differences between means were assessed using the least-significant difference (LSD) test. The validation process and the assumptions of analyses of variance followed using Shappiro–Wilk's W and Levenne tests to check the normal distribution and homogeneous variance, respectively. When non-compliance with ANOVA assumptions was observed, data transformation followed, using angular transformations ($\arcsin x^{0.5}$) of data expressed as percentages.

The relationship between visible injury records and the different O_3 exposure indices was analysed using Pearson's correlation index and the level of significance

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[1] UN ECE CLRTAP stands for United Nations/Economic Commission for Europe Convention on Long-Range Transboundary Air Pollution.

Table 1
Experimental protocol and ozone exposure related with the induction of foliar visible injury on the Papilionaceae species involved in the screening experiment

Papilionaceae	Plant entrance in OTC	Total exposure length (days)	NFA				NFA+			
			Time length injury develop[a] (days)	AOT40 until injury[b] (nl l^{-1} h)	AOT40 VPD > 1.5[c] (5 days) (nl l^{-1} h)	AOT40 VPD < 1.5[d] (5 days) (nl l^{-1} h)	Time length injury develop[a] (days)	AOT40 until injury[b] (nl l^{-1} h)	AOT40 VPD > 1.5[c] (5 days) (nl l^{-1} h)	AOT40 VPD < 1.5[d] (5 days) (nl l^{-1} h)
Anthyllis cornicina L.	07/09/00	76	—	—	—	—	—	15,189[e]	—	—
Anthyllis lotoides L.	14/09/00	69	—	—	—	—	—	13,144[e]	—	—
Biserrula pelecinus L.	22/09/00	67	67	313	0	0	28	5698	0	1086
Medicago minima (L.) Bartal	11/10/00	43	—	—	—	—	28	3942	0	788
Ornithopus compressus L.	11/10/00	66	—	—	—	—	—	12,099[e]	—	—
Trifolium angustifolium L.	11/10/00	43	28	67	0	0	28	3942	0	788
Trifolium cherleri L.	08/09/00	77	5	359	17	342	5	1432	186	1247
Trifolium glomeratum L.	08/09/00	68	5	359	17	342	5	1432	186	1247
Trifolium hirtum All.	22/09/00	47	47	288	0	0	13	2956	108	711
Trifolium striatum L.	14/09/00	61	1	17	0	301	1	226	0	1776
Trifolium subterraneum L.	08/09/00	66	5	359	17	342	5	1432	186	1247

[a] Time length for the detection of the first visible injury.

[b] Accumulated ozone exposure over 40 nl l^{-1} (AOT40) from the start of the experiment until the observation of the first foliar injury.

[c] Integrated ozone exposure 5 days prior to the detection of the first foliar injury when air vapour pressure deficit (VPD) was above 1.5 kPa, respectively, for the non-filtered air (NFA) or non-filtered air supplemented with 40 nl l^{-1} O_3 treatments (NFA+).

[d] Integrated ozone exposure 5 days prior to the detection of the first foliar injury when air vapour pressure deficit (VPD) was below 1.5 kPa, respectively, for the non-filtered air (NFA) or non-filtered air supplemented with 40 nl l^{-1} O_3 treatments (NFA+).

[e] Ozone exposure throughout the experiment, no visible injury was found in these species.

Table 2
Experimental protocol and ozone exposure related with the induction of foliar visible injury on the Poaceae species involved in the screening experiment

Poaceae	Plant entrance in OTC	Total exposure length (days)	NFA				NFA+			
			Time length injury develop[a] (days)	AOT40 until injury[b] ($nl\,l^{-1}\,h$)	AOT40 VPD > 1.5[c] (5 days) ($nl\,l^{-1}\,h$)	AOT40 VPD < 1.5[d] (5 days) ($nl\,l^{-1}\,h$)	Time length injury develop[a] (days)	AOT40 until injury[b] ($nl\,l^{-1}\,h$)	AOT40 VPD > 1.5[c] (5 days) ($nl\,l^{-1}\,h$)	AOT40 VPD < 1.5[d] (5 days) ($nl\,l^{-1}\,h$)
Aegilops geniculata Roth	07/09/00	71	43	776	0	47	28	7002	108	711
Aegilops triuncialis L.	07/09/00	72	—	—	—	—	28	7002	108	711
Avena barbata Link	11/10/00		28	67	0	0	9	1480	0	1086
Avena sterilis L.	03/10/00	49	35	160	0	0	17	3411	0	1086
Briza maxima L.	20/09/00	63	—	—	—	—	28	8934	0	788
Bromus hordeaceus L.	07/09/00	69	—	—	—	—	61	12,206	108	711
Bromus sterilis L.	14/09/00	67	—	—	—	—	54	10,160	0	788
Cynosurus echinatus L.	14/09/00	68	—	—	—	—	—	12,794[e]	—	—
Lolium rigidum Gaudin	11/10/00	43	—	—	—	—	9	1480	0	1086

[a] Time length for the detection of the first visible injury.

[b] Accumulated ozone exposure over $40\,nl\,l^{-1}$ (AOT40) from the start of the experiment until the observation of the first foliar injury.

[c] Integrated ozone exposure 5 days prior to the detection of the first foliar injury when air vapour pressure deficit (VPD) was above 1.5 kPa, respectively, for the non-filtered air (NFA) or non-filtered air supplemented with $40\,nl\,l^{-1}$ O_3 treatments (NFA+).

[d] Integrated ozone exposure 5 days prior to the detection of the first foliar injury when air vapour pressure deficit (VPD) was below 1.5 kPa, respectively, for the non-filtered air (NFA) or non-filtered air supplemented with $40\,nl\,l^{-1}$ O_3 treatments (NFA+).

[e] Ozone exposure throughout the experiment, no visible injury was found in these species.

was determined using the Student's *t*-test. All statistical analyses were carried out using Statistica 5.1. software.

3. Results and discussion

Ozone induced the appearance of specific and senescence-related foliar symptoms in 16 of the 22 assessed species. Both families (Poaceae and Papilionaceae) presented O_3-sensitive species, although differences in the type of injury, the number of affected species and the extent of visible injury on their foliage were found between them.

Ozone-induced injury on Papilionaceae species consisted of brown-reddish necrotic spots that were associated with foliar chlorosis in the case of *T. angustifolium* and *Medicago minima*. The *Trifolium* genus was the most O_3-sensitive taxa since all the assessed species showed visible symptoms following short O_3 exposures under ambient or close to ambient concentrations, in agreement with the results reported by Nebel and Fuhrer (1994). This is a finding of concern, since it is the most predominant genus in the therophytic and siliceous pastures of the Iberian Peninsula (Allué Andrade and Tella, 1986).

The O_3 sensitivity ranking of the assessed Papilionaceae species was: *T. striatum* > *T. cherleri, T. glomeratum, T. subterraneum, T. hirtum* > *T. angustifolium* > *Biserrula pelecinus, M. minima* > *Anthyllis lotoides, A. cornicina, O. compressus*.

T. striatum was the most sensitive species of this genus; O_3-induced injury was observed in the first fully expanded trifoliate even before the plants were introduced in the chambers, associated with a very low AOT40 value, $17\,\mathrm{nl\,l^{-1}\,h}$ (Table 1). *T. cherleri, T. glomeratum, T. subterraneum* and *T. hirtum* were rather sensitive to O_3 exposure since the first O_3 visible injury was found with AOT40 values ranging $288–359\,\mathrm{nl\,l^{-1}\,h}$ (Table 1). These values are much lower than those reported by Bergmann et al. (1999) for 25 species of German flora, ca. $2000\,\mathrm{nl\,l^{-1}\,h}$. In fact, when the O_3 exposure corresponding to the 5 days prior to the first observation of injury was considered, the AOT40 values ranged from 0 to 17 when VPD > 1.5 kPa and from 0 to 342 when VPD < 1.5 kPa. These values are lower than the $200–500\,\mathrm{nl\,l^{-1}}$ range reported for the induction of visible injury on a pan-European study involving *T. subterraneum* (Benton et al., 2000). A gradation in foliar injury (both O_3-induced visible injury and senescent leaves) was found in the Papilionaceae group when plants were exposed to the different treatments for 66–77 days (Fig. 1); the extent of visible injury increased ($p<0.05$) in NFA and NFA+ plants by 45–60% and 75–85%, respectively, when compared with CFA plants.

The first foliar injury on *T. angustifolium* was found at a rather low AOT40 value ($67\,\mathrm{nl\,l^{-1}\,h}$); however, injury developed at a low rate after 43 days of exposure and only affected 10% of the foliage of NFA+ plants (Table 1, Fig. 1). Ozone exposure did not induce an increase in senescence-related symptoms. This behaviour would indicate a greater sensitivity of early stages of development when compared to late phenological stages. Franzaring et al. (2000) also reported more resistant and robust leaves in the later stages, while Carlsson et al. (1996) found an inverse relationship. However, in this species, O_3 exposure appears to be related with the induction and extension of interveinal chlorosis in the trifoliates (not quantified). This type of injury was not found in any other of the assessed *Trifolium* species and is considered as a non-specific O_3 injury.

B. pelecinus and *M. minima* could be considered as less sensitive than *Trifolium* species since ambient O_3 levels induced foliar injury *B. pelecinus* only at the end of the experiment and did not determine any effect on *M. minima*. Moreover, the first foliar injury was observed in the NFA+ plants of both species after 28 days of exposure in association with AOT40 values in the 3942–5698 $\mathrm{nl\,l^{-1}\,h}$ range (Table 1). *B. pelecinus* would be slightly more sensitive than *M. minima* since after 67 days of exposure O_3-induced injury was found in 20% of the leaves of NFA and NFA+ plants and an increment of senescence in NFA+ leaves was also found ($p<0.05$) (Fig. 1).

A. cornicina, A. lotoides and *O. compressus* can be considered as the most resistant species to O_3 exposure as no effects were detected on their leaves after an average exposure length of 70 days, with AOT40 values up to $12{,}000–15{,}000\,\mathrm{nl\,l^{-1}\,h}$ in the NFA+ treatment (Table 1). Foliage was apparently healthy and O_3 exposure did not cause an increased senescence in these species (Table 1).

The O_3-induced foliar injury observed in the species of the Poaceae family was different from that observed in the Papilionaceae species. Apical necrosis and reddish-brown spots parallel to leaf's nerves were observed in *Avena sterilis* and *A. barbata*. Apical necrosis followed by a chlorotic banding parallel to leaf's nerves was found in *Aegilops geniculata* and *A. triuncialis*. Ozone exposure induced in 67% of Poaceae species an increase in all the senescent-related parameters assessed: number of senescent leaves to total leaves ratio, dry weight of total senescent foliage per plant or green biomass to senescent biomass ratio. Therefore, senescence appears to be a crucial parameter to determine the O_3 sensitivity of the dehesa therophytic species of the Poaceae family.

Also, the grass species were classified regarding their O_3 sensitivity considering the O_3 exposure needed for the induction of foliar visible injury symptoms: *A. barbata, A. sterilis, A. geniculata* > *Lolium rigidum* > *Briza maxima, A. triuncialis, Bromus hordeaceus, B. sterilis* > *Cynosurus echinatus, Micropyrum tenellum, Vulpia myuros*.

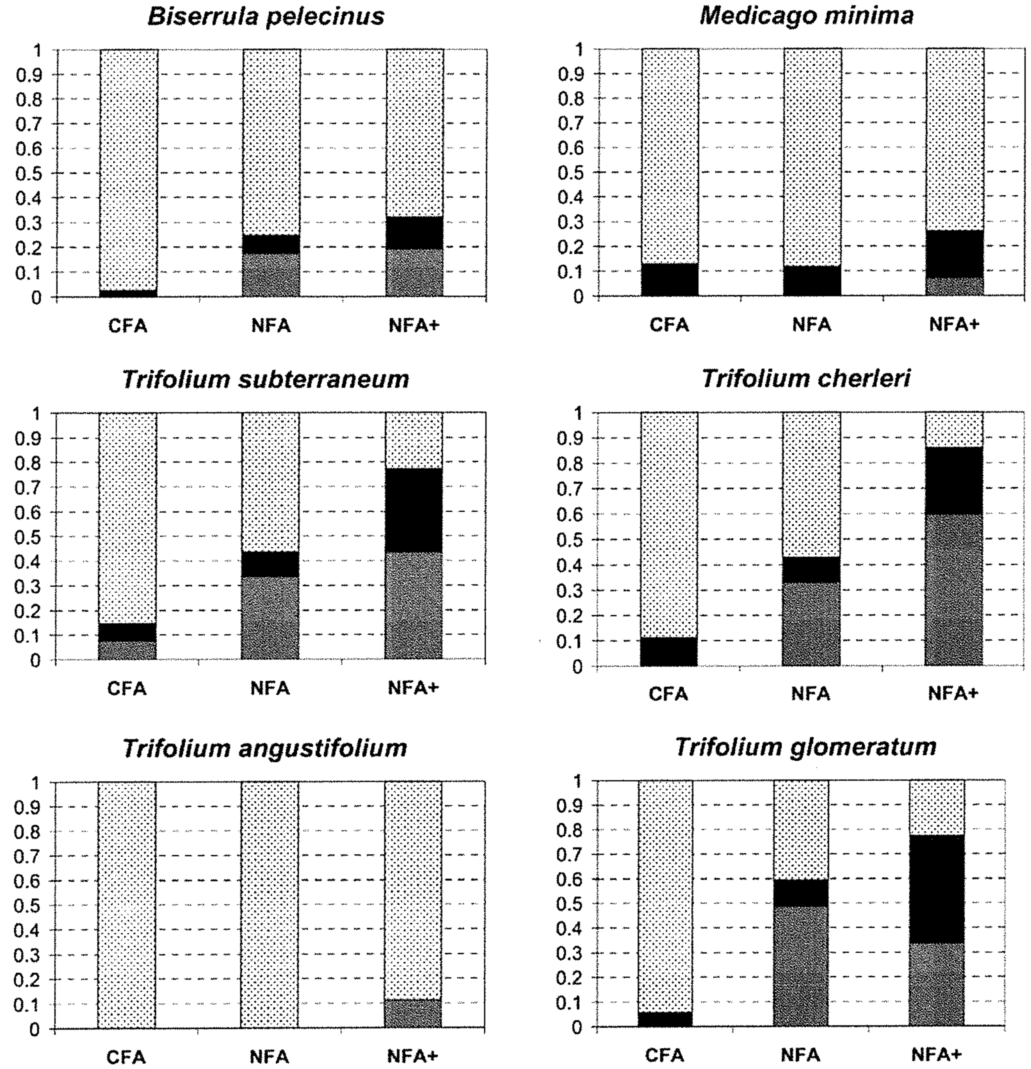

Fig. 1. Foliar injury (‰) of the Papilionaceae species involved in the experiment. ▨ = ozone-specific visible symptoms; ■ = senescence-related injury; ░ = healthy foliage. CFA = charcoal-filtered air; NFA = non-filtered air; NFA + = non-filtered air supplemented with 40 nl l^{-1} ozone.

A. barbata, *A. sterilis* and *A. geniculata* would be the most sensitive grasses since O_3 foliar injury was observed after 28–43 days of ambient O_3 exposure corresponding to AOT40 values ranging 67–776 nl l^{-1} h (Table 2). At the end of the experiment, ambient O_3 levels induced specific visible symptoms on about 20% of the leaves of *A. sterilis and A. geniculata* compared to control ($p<0.05$) (Fig. 2). In the case of *A. geniculata*, ambient O_3 levels also determined a 20% increase in foliar senescence ($p<0.01$).

Foliar injury was also observed in other species, but a greater accumulated O_3 exposure was needed to trigger visible injury on their foliage. For instance, the first foliar injury in *L. rigidum* was found in leaves of NFA + plants exposed to an AOT40 value of 1480 nl l^{-1} h (Table 2). At the end of the experiment, O_3 foliar damage increased in NFA and NFA + compared to control ($p<0.01$), but no differences between NFA and NFA + plants with regard to the number of O_3-affected leaves were found. However, a significant gradation in the number of senescent leaves from the different O_3 treatments was observed in this species ($p<0.01$) (Fig. 2).

The group constituted by *A. triuncialis*, *B. hordeaceus*, *B. sterilis* and *B. maxima* could be considered as less sensitive since the first O_3-induced visible injury was found following AOT40 exposures ranging 7000–12,200 nl l^{-1} h (Table 2). When the extension of visible injury was analysed at the end of the experiment, *B. maxima* was the most sensitive species within this group since the NFA and NFA + treatments induced a similar extent of injury on its foliage (Fig. 2). *A. triuncialis*

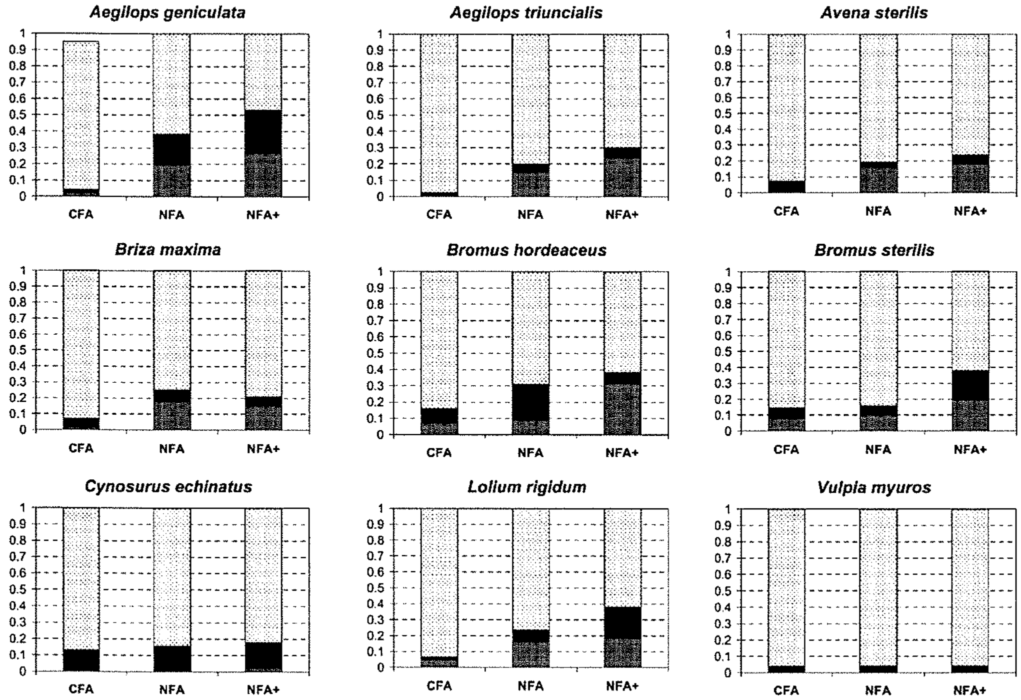

Fig. 2. Foliar injury (‰) of the Poaceae species involved in the experiment. ▩ = ozone-specific visible symptoms; ■ = senescence-related injury; ▒ = healthy foliage. CFA = charcoal-filtered air; NFA = non-filtered air; NFA + = non-filtered air supplemented with 40 nl l^{-1} ozone.

would be the next sensitive species with a marked gradation in the development of foliar injury between the different treatments ($p<0.05$). An increase in the extension of O_3 foliar injury was found only in those *B. hordeaceus* plants exposed to the NFA+ treatment when compared to control plants ($p<0.05$) (Fig. 2). An increase in senescence following O_3 exposure in NFA+ was observed in *B. sterilis* ($p<0.05$), an effect that was not observed in the other species of this group. The increase in the number of senescent leaves is an unspecific but characteristic effect of this pollutant on natural vegetation (Bergmann et al., 1999; Franzaring et al., 2000). In fact, the ratio between the number of senescent leaves to total leaves recorded at the end of the experiment was more sensitive than the induction of specific symptoms following the exposure of *B. sterilis* individuals to increasing O_3 concentrations.

The most O_3-resistant grass species regarding visible injury were *C. echinatus*, *M. tenellum* and *V. myuros*, species that did not show any effect on their foliage, either as specific O_3 symptoms or as senescence-related injury (Table 2).

The Papilionaceae species assessed in the experiment were more sensitive to O_3 than the Poaceae species. Ambient O_3 exposures involving AOT40 values close to 300 nl l^{-1} h and relatively short time lengths induced foliar visible symptoms in 67% of Papilionaceae species. By contrast, ambient O_3 exposures determined the appearance of foliar symptoms in only 27% of Poaceae plants, and usually O_3 exposures with AOT40 values over 1000 nl l^{-1} h were needed to induce foliar injury in the majority of the species of this family (Tables 1 and 2).

When the proportion of healthy, senescent and injured leaves was considered, O_3 impact was the greatest in the Papilionaceae family as revealed by the two-way ANOVA analysis considering family and ozone as factors (Table 3). The extent of visible injury was influenced by both family and O_3 treatment ($p<0.0001$). An O_3-family interaction ($p<0.05$) was found for the percentage of senescent leaves, since the NFA+ Papilionaceae plants presented an increase of 37% of senescent leaves when compared with the Poaceae plants of the same treatment.

When overall data were analysed, O_3-induced foliar injury was recorded in association with O_3 exposures well below current O_3 short-term critical levels (see Tables 1 and 2). This was especially true when air VPD was higher than 1.5 kPa. Under these conditions, O_3 visible injury was found associated with AOT40 values below the proposed 500 nl l^{-1} h O_3 threshold, in the ranges from 0–17 to 0–186 nl l^{-1} h for the most sensitive species when exposed to ambient or artificially supplemented O_3 levels respectively. Therefore, the present O_3 critical level for visible injury does not appear to protect the species from dehesa ecosystems. This conclusion is in agreement with the findings reported by Tonneijck and Van Dijk (2002) for *T. subterraneum*.

Table 3
Extent of foliar injury in Papilionaceae and Poaceae taxa at the end of the exposure period

Factor	% of leaves showing specific O_3 injury	% of leaves showing senescence-related injury	% of healthy leaves
O_3	<0.0001	ns	<0.0001
Family	<0.0001	<0.0001	<0.0001
O_3 × Family	ns	<0.05	<0.1
Papilionaceae			
CFA	0.09 ± 0.04	0.06 ± 0.01^{a}	0.86 ± 0.04
NFA	0.30 ± 0.05	0.07 ± 0.01^{a}	0.63 ± 0.05
NFA+	0.32 ± 0.04	0.19 ± 0.03^{c}	0.48 ± 0.06
Poaceae			
CFA	0.03 ± 0.01	0.06 ± 0.01^{a}	0.90 ± 0.01
NFA	0.11 ± 0.01	0.09 ± 0.01^{ab}	0.79 ± 0.02
NFA+	0.17 ± 0.02	0.12 ± 0.01^{b}	0.71 ± 0.03

Different letters indicate significant differences among ozone treatments. CFA = charcoal-filtered air; NFA = non-filtered air; NFA+ = non-filtered air supplemented with 40 nl l^{-1} O_3. Mean values ± standard errors.

The extent of O_3-induced injury on plant foliage was more related with plant performance than the appearance of the first visible injury. Correlation analyses between the extent of foliar injury in Papilionaceae or Poaceae plants and different O_3 exposure indices were carried out. Only significant relationships ($p<0.05$) are commented below. The average exposure indices, such as M24 or M10, and indices based on a cut-off of 30 nl l^{-1}, such as AOT30 or NUM30, performed better than AOT40 index in explaining the extent of visible injury on the assessed plant species. The performance of these indices depended on the type of injury. The extent of specific O_3 injury was best explained ($r = 0.85$) by NUM30 in the Papilionaceae plants, while it was almost equally explained by M10, M24, NUM30 or maximum hourly values ($0.62<r\leqslant 0.65$) in the Poaceae species.

Interestingly, the NUM30 ($r = 0.85$) and NUM40 ($r = 0.70$) indices performed better than the AOT30 ($r = 0.66$) and AOT40 ($r = 0.60$) indices, respectively, in explaining the extent of O_3-specific injury on the foliage of Papilionaceae plants. This would indicate that all concentrations above a given threshold are equally phytotoxic since the AOT-type indices implicitly provide more weight to the higher O_3 concentrations (Tuovinen, 2000) and would be related with the high O_3 sensitivity of this taxon.

The extent of leaf senescence in Papilionaceae plants was almost equally explained by AOT30, AOT40, NUM40, AOT60 or NUM60 indices ($0.74<r<0.76$). In the case of Poaceae plants, this parameter was also almost equally explained by NUM30, AOT30, M24, M10, maximum hourly values and NUM40 ($0.37<r<0.40$).

The number of hours where O_3 concentrations were above the cut-off of 30 nl l^{-1} (NUM30) was the index that best explained the total extent of O_3-induced foliar injury (specific O_3 injury plus senescent foliage) in both Papilionaceae ($r = 0.87$) and Poaceae species ($r = 0.68$). Regression equations were constructed separately to predict the response of both families to O_3: % affected leaves in Papilionaceae plants = 0.0911 NUM30 ($R^2 = 0.76$) and % affected leaves in Poaceae plants = 0.0369 NUM30 ($R^2 = 0.47$). Exposures to 55 and 110 h over 30 nl l^{-1} would be needed to determine 5% and 10% injury on the foliage of Papilionaceae species, respectively. Similarly, 5% and 10% foliage damage on Poaceae species would appear when exposed to 136 and 271 h over 30 nl l^{-1} of O_3.

Since visible injury was observed in association with zero AOT40 values accumulated during the 5 days preceding the detection of O_3 injury, our results suggest that a cut-off lower than 40 nl l^{-1} should be considered for adequate plant protection. This is in agreement with the findings of Pihl Karlsson et al. (1995) for *T. subterraneum* and Ribas and Peñuelas (2003) for tobacco cv. Bel-W3. A meta-analysis is envisaged to ascertain whether this proposal is valid when data from pan-European observations and experimentation are analysed.

4. Conclusions

The results of our experiments indicate the great O_3 sensitivity of the therophytic plants from dehesa ecosystems based on the induction of O_3 visible injury. The Papilionaceae plants, particularly those belonging to the *Trifolium* genus, were more sensitive than the Poaceae species. Ozone induced both symptomatic and asymptomatic (senescence-related) foliar injury on some species at exposures well below current short-term critical levels. Therefore, indices based on a cut-off of $30\,\mathrm{nl\,l^{-1}}$ instead of current $40\,\mathrm{nl\,l^{-1}}$ threshold are proposed. These species could be useful in future O_3 bioindication programmes focused in the Mediterranean area.

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References

Alonso, R., Elvira, S., Castillo, F.J., Gimeno, B.S., 2001. Interactive effects of ozone and drought stress on pigments and activities of oxidative enzymes in *Pinus halepensis*. Plant Cell and Environment 24, 905–916.

Allué Andrade, J.L., Tella, G., 1986. Los pastos españoles. II. Clasificación, composición, atributos y mejora de sus principales tipos florísticos. E.U.I.T. Forestal, Madrid, Spain.

Ashmore, M.R., Dalpra, C., Tickle, A.K., 1987. Effects of ozone and calcium nutrition on native plant species. In: Mathy, P. (Ed.), Air pollution and ecosystems. Reidel, Dordrecht, The Netherlands, pp. 647–652.

Ashmore, M.R., Thwaites, R.H., Aisnworth, N., Cousins, D.A., Power, S.A., Morton, J., 1995. Effects of ozone on calcareous grassland communities. Water, Air and Soil Pollution 85, 1527–1532.

Benton, J., Fuhrer, J., Gimeno, B.S., Skärby, L., Palmer-Brown, D., Ball, G., Roadknight, C., Mills, G., 2000. An international co-operative programme indicates the widespread occurrence of ozone injury on crops. Agriculture, Ecosystems and Environment 78, 19–30.

Bermejo, V., Gimeno, B.S., Granados, I., Santamaría, J., Irigoyen, J.J., Bermejo, R., Porcuna, J.L., Mills, G., 2002. Investigating indices to explain the impacts of ozone on the biomass of white clover (*Trifolium repens* L. cv. Regal) at inland and coastal sites in Spain. New Phytologist 156, 43–55.

Bergmann, E., Bender, J., Weigel, H-J., 1999. Ozone threshold doses and exposure–response relationships for the development of ozone injury symptoms in wild plant species. New Phytologist 144, 423–435.

Carlsson, A.S., Wallin, G., Sandelius, A.S., 1996. Species- and age-dependent sensitivity to ozone in young plants of pea, wheat and spinach, effects on acyl lipid and pigment content and metabolism. Physiologia Plantarum 98, 271–280.

Davison, A.W., Barnes, J.D., 1998. Effects of ozone on wild plants. New Phytologist 139, 135–151.

EMEP, 2002.Transboundary acidification, eutrophication and ground level ozone in Europe. EMEP Report 1 and 2/2002. EMEP, Oslo, Norway.

Franzaring, J., Tonneijck, A.E.G., Koijman, A.W.N., Dueck, Th.A., 2000. Growth responses to ozone in plant species from wetlands. Environmental and Experimental Botany 44, 39–48.

Fuhrer, J., Acherman, B. (Eds.), 1999. Critical Levels for Ozone—Level II. Environmental Documentation 115. Swiss Agency for Environment, Forests and Landscape, Bern, Switzerland.

Fuhrer, J., Shariat-Madari, H., Perler, R., Tschannen, W., Grub, A., 1994. Effects of ozone on managed pasture. II. Yield, species composition, canopy structure and forage quality. Environmental Pollution 83, 317–325.

Fumagalli, I., Gimeno, B.S., Velissariou, D., Mills, G., De Temmerman, L., Fuhrer, J., 2001. Evidence of ozone-induced adverse effects on Mediterranean vegetation. Atmospheric Environment 35, 2583–2587.

Gimeno, B.S., Bermejo, V., Reinert, R.A., Zheng, Y., Barnes, J.D., 1999. Adverse effects of ambient ozone on watermelon yield and physiology at a rural site in Eastern Spain. New Phytologist 144, 245–260.

Madkour, S.A., Laurence, J.A., 2002. Egyptian plant species as new ozone indicators. Environmental Pollution 120, 339–353.

Marañon, T., 1985. Diversidad florística y heterogeneidad ambiental en una dehesa de Sierra Morena. Anales de Edafología y Agrobiología 44, 1183–1197.

Myers, N., Mittermeier, R.A., Mittermeier, C.G., da Fonseca, C.A.B., Kent, J., 2000. Biodiversity hotspots for conservation priorities. Nature 403, 853–858.

Nali, C., Pucciariello, C., Lorenzini, G., 2002. Ozone distribution in central Italy and its effect on crop productivity. Agriculture, Ecosystems and Environment 90, 277–289.

Nebel, B., Fuhrer, J., 1994. Inter- and intra-specific differences in ozone sensitivity in semi-natural plant communities. Angewandte Botanik 68, 116–121.

Peco, B., Espigares, T., Levassor, C., 1998. Trends and fluctuations in species abundance and richness in Mediterranean annual pastures. Applied Vegetation Science 1, 21–28.

Pihl Karlsson, G., Pleijel, H., Sild, E., Danielsson, H., Selldén, G., Ericson, L., Skärby, L., 1995. Clover Sweden—a national three year study of the effects of troposheric ozone on *Trifolium subterraneum*. Water, Air and Soil Pollution 85, 1503–1508.

Pineda, F.D., Nicolás, J.P., Ruiz, M., Peco, B., Bernáldez, F.G., 1981. Succesion, diversité et amplitude de niche dans les aturages du centre de la péninsule ibérique. Vegetatio 47, 267–277.

Plaza, J., Pujadas, M., Artíñano, B., 1997. Formation and transport of the Madrid ozone plume. Journal of Air and Waste Management Association 47, 666–774.

Pleijel, H., Danielsson, H., 1997. Growth of 27 herbs and grasses in relation to ozone exposure and plant strategy. New Phytologist 135, 361–367.

Pujadas, M., Terés, J., Gimeno, B.S., 1997. La experiencia española en el diseño de sistemas experimentales para el estudio de efectos producidos por contaminantes gaseosos sobre especies vegetales. Boletín de Sanidad Vegetal—Plagas 23, 39–54.

Ribas, A., Peñuelas, J., 2003. Biomonitoring of tropospheric ozone phytotoxicity in rural Catalonia. Atmospheric Environment 37, 63–71.

Sala, O.E., Chapin III, F.J., Armesto, J.J., Berlow, E., Bloomfield, J., Dirzo, R., Huber-Sanwald, E., Huenneke, L., Jackson, R.B., Kinzig, A., Leemans, R., Lodge, D., Mooney, H., Oesterheld, M., Poff, N.L., Sykes, M.T., Walker, B.H., Walker, M., Wall, D.H., 2000. Global diversity scenarios for the year 2100. Science 287, 1770–1774.

San Miguel, A., 1994. La dehesa española. Origen, tipología, características y gestión. E.T.S.I. Montes, Madrid, Spain.

Tonneijck, A.E.G., Van Dijk, C.J., 2002. Injury and growth response of subterranean clover to ambient ozone as assessed by using ethylendiurea (EDU): three years of plant monitoring at four sites in the Netherlands. Environmental and Experimental Botany 48, 33–41.

Tuovinen, J.P., 2000. Assessing vegetation exposure to ozone: properties of the AOT40 index and modifications by deposition modelling. Environmental Pollution 109, 361–372.

UN ECE, 1988. ECE Critical Levels Workshop. BadHarzburg14–18 March 1988. Final Draft Report. Unwelbundesamt, Berlin, Germany.

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Growth response to ozone of annual species from Mediterranean pastures

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"Capsule": *The therophytes from dehesa acidic pastures of central of the Iberian peninsula present a great sensitivity to ozone, as derived from growth- and biomass-related variables.*

Abstract

Ozone (O_3) phytotoxicity has been reported on a wide range of plant species. However, scarce information has been provided regarding the sensitivity of semi-natural grassland species, especially those from dehesa Mediterranean grasslands, in spite of their great biological diversity and the high O_3 levels recorded in the region. A screening study was carried out in open-top chambers (OTCs) to assess the O_3-sensitivity of representative therophytes of these ecosystems based on the response of selected growth-related parameters. Three O_3 treatments and 3 OTCs per treatment were used. Legume species were very sensitive to O_3, because 78% of the tested species showed detrimental effects on their total biomass relative growth rate (RGR) following their exposure to O_3. The *Trifolium* genus was particularly sensitive showing O_3-induced adverse effects on most of the assessed parameters. Gramineae plants were less sensitive than Leguminosae species because detrimental effects on total biomass RGR were only observed in 14% of the assessed species. No relationship was found between relative growth rates when growing in clean air and O_3 susceptibility. The implications of these effects on the performance of dehesa acidic grasslands and on the definition of ozone critical levels for the protection of semi-natural vegetation are discussed.

Keywords: Relative growth rate; Dehesa; Screening; Therophytes; Critical levels

1. Introduction

Semi-natural vegetation is frequently exposed to elevated ozone (O_3) concentrations throughout Europe (EMEP, 2002) which can adversely affect the most sensitive species, inducing changes in the floristic composition of plant communities (Ashmore and Ainsworth, 1995; Barbo et al., 1998; Fuhrer et al., 1994). However, limited information has been provided regarding the sensitivity of these ecosystems to O_3 as has been recently reported in the last UN/ECE (United Nations Economic Commission for Europe) workshop held to define ozone critical levels for plant protection (Ashmore and Franzaring, 2003). As a result, O_3 critical levels for semi-natural vegetation are poorly defined, especially for the Mediterranean area where high O_3 concentrations have been recorded.

Few studies have been carried out aiming to evaluate the O_3 sensitivity of Mediterranean grassland species. For instance, Velissariou and Davison (1994), Madkour and Laurence (2002) and El-Khatib (2003) reported the great sensitivity of several local Greek and Egyptian

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cultivars of fodder crops, the Leguminosae species *Medicago sativa* and *Trifolium alexandrinum* being the most sensitive ones of those assessed. More recently, Bermejo et al. (2003) and Gimeno et al. (2004) have carried out experiments focused on the therophytic pastures from dehesa acidic grasslands of the central Iberian peninsula. These are valuable ecosystems presenting a great biodiversity (Pineda et al., 2002). In these pastures therophytes are more predominant than perennial species, representing up to 72% of the species (Azcarate et al., 2002). The screening experiment carried out by Bermejo et al. (2003) to assess the sensitivity of 22 annual species from these ecosystems based on the detection of foliar visible injury indicated that the legumes, especially the assessed *Trifolium* species, were more sensitive to O_3 than grasses. This differential sensitivity might affect the structure and function of these ecosystems and also could modify the nutritive quality of the pastures for the herbivores. Several experiments have also indicated a greater O_3 sensitivity of Leguminosae species when compared with Gramineae plants (Nussbaum et al., 1995; Warwick and Taylor, 1995).

Plant sensitivity to this pollutant is usually related to the induction of foliar visible injury. However, the use of foliage health as the main criterion to assess potential O_3 adverse effects may not be the best indicator of plant performance due to the weak association between visible injury and other important parameters, such as plant growth, which are more fundamental for plant survival and reproduction (Davison and Barnes, 1998; Reiling and Davison, 1992). Nevertheless, the immense number of wild species makes it essential that species can be ranked and the most sensitive taxa identified for the definition of O_3 critical levels (Davison and Barnes, 1998).

To overcome the above-mentioned gaps in knowledge a screening study involving 19 characteristic therophytes of the dehesa acidic grasslands was carried out to falsify the hypothesis that no differences in the growth responses of annual legumes and grasses to ozone exposure should be expected. The following specific objectives of the study were defined: (1) to assess their O_3-sensitivity based on plant growth-related parameters, (2) to rank the sensitivity of the selected species and (3) to test whether plant O_3 sensitivity can be linked to its classification within a given taxon.

2. Materials and methods

2.1. Plant material

The 19 species involved in this study are characteristic therophytes of dehesa acidic grasslands of central Iberia peninsula (Montoya et al., 1988; San Miguel 1994). Most of the seeds were collected from a typical dehesa located northwards from Madrid (Dehesa de Moncalvillo, Guadalix de la Sierra, Madrid; 40°40′N 03°46′W). The germoplasm bank of the Agriculture and Environment Council from the Extremadura Autonomous Community supplied the *Trifolium striatum*, *Trifolium subterraneum*, *Trifolium angustifolium* and *Ornithopus compressus* seeds, collected at different sites from central-western areas of the Iberian peninsula. The Spanish *T. subterraneum* cv. Zujar was used in the experiments.

All seeds of Leguminosae species were immersed during 24 h in a Germinator© solution (Agro-Orgánicos Mediterráneos S.L., Granada, Spain) to ensure a homogeneous germination. When seeds were swollen they were sown in a 50% neutral peat and 50% vermiculite substrate. Gramineae seeds did not experience any pretreatment and they were sown in the same substrate as Leguminosae seeds. The seedlings were transplanted to 2.5 l pots with a 50% peat, 30% vermiculite and 20% perlite substrate; $2\,\mathrm{kg\,m^{-3}}$ of a slow-release fertilizer (NPK:15/8/11) was applied. Plants were irrigated with a droplet system to ensure adequate and homogeneous water availability to plant material. The species involved in the study and their sowing dates are presented in Table 1.

2.2. Ozone treatments

The assay was performed in an open-top Chamber (OTC) experimental field located at Sant Jaume d'Enveija, Spain (40°41′N, 0°47′E). The same day the plants were transplanted into pots they were introduced in slightly modified NCLAN-type OTCs (see Gimeno et al., 1999). Three O_3 treatments were used: charcoal filtered air (CFA) presenting subphytotoxic O_3 levels, non-filtered air (NFA) with close to ambient O_3 levels and non-filtered air supplemented with 40 ppb O_3 from 07:00 to 17:00 (GMT) 5 days week^{-1} (NFA+). Realistic O_3 levels were recorded in the latter treatment, similar to those experienced in the field by the selected species (Palacios et al., 2002; Plaza et al., 1997). Four to six plants from each species were introduced in the chambers and three OTC replicates were used for each O_3 treatment. An automatic system was used to provide a continuous monitoring of O_3, sulphur dioxide and nitrogen oxides concentrations in the different treatments, along with meteorological parameters such as wind speed and direction, air temperature and relative humidity and photosynthetic active radiation (PAR). A complete description of the chambers and the operation of the system is provided in Alonso et al. (2001).

The O_3 exposure index AOT40 currently used by both the UN/ECE CLRTAP[1] and the European daughter O_3 Directive (2002/3/EC) was calculated for each

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[1] UN/ECE CLRTAP. United Nations/Economic Commission for Europe Convention on Long-Range Transboundary Air Pollution.

Table 1
Time-table and ozone exposure corresponding to the assessment of the ozone sensitivity of dehesa therophytic species

	Sowing date	Plant entrance in OTC	First harvest date (day/month)	Last harvest date (day/month)	Total Exposure length (days)	AOT40 (ppb h)	
						NFA	NFA+
Leguminosae (legumes)							
Anthyllis cornicina L.	01/08/00	07/09/00	22/09	22/11	76	821	15,189
Anthyllis lotoides L.	01/08/00	14/09/00	15/09	22/11	69	415	13,144
Biserrula pelecinus L.	01/08/00	22/09/00	22/09	28/11	67	313	12,236
Medicago minima (L.) Bartal	02/09/00	11/10/00	09/09	23/11	43	92	7222
Ornithopus compressus L.	02/09/00	11/10/00	09/10	27/11	47	313	12,099
Trifolium angustifolium L.	02/09/00	11/10/00	09/10	23/11	43	92	7222
Trifolium cherleri L.	01/08/00	08/09/00	16/09	9/11	62	783	15,493
Trifolium glomeratum L.	01/08/00	08/09/00	20/09	8/11	61	780	13,578
Trifolium striatum L.	01/08/00	14/09/00	15/09	7/11	54	412	11,479
Trifolium subterraneum L.	01/08/00	08/09/00	15/09	13/11	66	775	12,988
Gramineae (grasses)							
Aegilops geniculata Roth	01/08/00	07/09/00	15/09	10/11	64	818	14,183
Aegilops triuncialis L.	01/08/00	07/09/00	16/09	11/11	65	818	14,570
Avena sterilis L.	02/09/00	03/10/00	05/10	21/11	49	185	8503
Briza maxima L.	01/08/00	20/09/00	21/09	22/11	63	327	11,917
Bromus hordeaceus L.	01/08/00	07/09/00	15/09	16/11	69	818	13,865
Bromus sterilis L.	01/08/00	14/09/00	15/09	20/11	67	415	12,550
Cynosurus echinatus L.	01/08/00	14/09/00	15/09	21/11	68	415	12,791
Lolium rigidum Gaudin	02/09/00	11/10/00	09/10	23/11	43	92	7222
Vulpia myuros (L.) C.C.Gmelin	01/08/00	20/09/00	21/09	22/11	63	327	11,917

species and O_3 treatment as the sum of the differences between O_3 hourly concentrations in ppb and 40 ppb for each hour when the concentration exceeds 40 ppb and solar radiation is above 50 W m^{-2}.

2.3. Growth-related parameters

Plant height, diameter, aerial and root biomass of plant material were determined when plants were exposed to the different O_3 treatments during 43–77 days, mostly depending on the timing of seed germination.

Plant height and diameter were used to estimate the canopy volume and surface of the plant species involved in the experiment. Two diameter estimations were carried out per plant, their mean value was used for subsequent statistical analyses. The aboveground biomass was harvested by excising the plants at the surface of the substrate. Leaves and stems of Leguminosae plants were separated and dried at 60 °C until constant weight and subsequently weighed to determine their foliage/stem dry weight ratio. A similar procedure was followed for Gramineae species. Similarly, roots were cautiously extracted, washed, dried at 60 °C and weighed afterwards. The shoot/root ratios were also calculated to evaluate potential shifts in photosynthate distribution. Prior to the entrance of plant material in the OTCs the mean of the initial weights of the root and shoot biomass of an extra-set of 10 plants per species was used to derive the relative growth rates (RGR, g week^{-1}) of the experimental plants. The RGRs for aerial, root, and total biomass were calculated using the formula:

$$\mathrm{RGR} = \ln W_2 \ln W_1/t_2\ t_1$$

where W_2 and W_1 are the dry weights at the final harvest and at the harvest performed prior to the entrance of plant material in the chamber, respectively, and t_2 and t_1 are the number of days of both harvests (Hunt, 1990). Details of the dates when the plants were introduced in the chambers and the length of O_3 exposure in the different treatments can be found in Table 1.

2.4. Statistical analyses

An ANOVA analysis for each parameter and species was performed to evaluate O_3 effects on plant growth-related parameters. Also, a combined analysis involving all the assessed species was performed to assess whether plant sensitivity to O_3 exposure could be related to plant family; therefore a two-way ANOVA analyses were carried out considering ozone exposure and family as factors. When significant differences ($p < 0.05$) were detected, the differences between means were assessed using the least significant difference (LSD) test. Normal probability plots and scatter plots of residuals were used to test data normality. Levenne tests (Milliken and Johnson, 1994) were applied to check the variance homocedasticity. When necessary logarithmic or square-root transformations were used. The relationship between growth parameters and the different O_3 exposure indices was analysed using Pearson's correlation index and the level of significance was determined using the student *t*-test. All statistical analyses were carried out using Statistica 97 software.

3. Results and discussion

Of the 19 species assessed in this study, only *Anthilllis cornicina* could be considered as resistant to O_3 exposure since it was not significantly affected by any of the assessed parameters on this species. The growth or the foliar morphology of the remaining species was adversely affected by those concentrations of the pollutant which are actually recorded in the dehesas of the central Iberian peninsula (Palacios et al., 2002; Plaza et al., 1997). However, the nature of the effect differed depending on species and family.

In general, the Leguminosae species were more sensitive to O_3 than Gramineae species regarding the response of the assessed growth-related parameters as shown in Tables 2–4. Ozone exposure induced significant detrimental effects on the aerial and root RGRs of 70%

Table 2
Plant growth-related parameters of the legume grown in the different O_3 treatments

	Foliage dw (g)	Stem dw (g)	Root RGR	Aerial RGR	Total RGR	Foliage/stem	Shoot/root
Anthyllis cornicina L.							
CFA	1.91	0.26	0.26	0.40	0.38	8.88	10.27
NFA	1.82	0.26	0.28	0.39	0.37	6.93	7.17
NFA+	2.44	0.50	0.25	0.43	0.40	5.50	12.02
Anthyllis lotoides L.							
CFA	9.07	2.32	–	0.54	–	4.25	–
NFA	9.52	2.25	–	0.55	–	4.43	–
NFA+	8.42	2.01	–	0.54	–	5.07	–
Biserrula pelecinus L.							
CFA	9.15^{a}	4.53	0.34	0.36^{b}	0.36^{a}	2.07	4.76
NFA	7.00^{ab}	3.76	0.33	0.34^{ab}	0.34^{ab}	1.86	3.20
NFA+	5.16^{b}	2.39	0.31	0.29^{a}	0.29^{b}	2.13	2.66
Medicago minima (L.) Bartal							
CFA	0.95^{a}	0.47^{a}	0.41^{a}	0.46^{b}	0.44^{a}	2.07^{a}	2.27
NFA	0.56^{b}	0.30^{b}	0.39^{a}	0.44^{b}	0.42^{a}	1.87^{ab}	2.41
NFA+	0.45^{b}	0.27^{b}	0.32^{b}	0.37^{a}	0.35^{b}	1.64^{b}	2.33
Ornithopus compressus L.							
CFA	3.07	0.52	0.51	0.75^{a}	0.65	5.99	3.06^{a}
NFA	2.52	0.39	0.50	0.72	0.61	6.58	2.72^{a}
NFAA+	2.44	0.38	0.52	0.72	0.62	6.61	2.18^{b}
Trifolium angustifolium L.							
CFA	1.43^{a}	0.17^{a}	0.54^{a}	0.59^{b}	0.56^{a}	8.98	1.86
NFA	1.32^{a}	0.15^{a}	0.53^{a}	0.57^{b}	0.56^{a}	9.23	1.73
NFA+	0.88^{b}	0.10^{b}	0.46^{b}	0.51^{a}	0.49^{b}	10.57	1.89
Trifolium cherleri L.							
CFA	6.16^{a}	0.87	0.36^{a}	0.51^{b}	0.48^{a}	5.88	7.66^{a}
NFA	3.19^{ab}	1.03	0.36^{a}	0.46^{ab}	0.43^{ab}	3.22	4.93^{b}
NFA+	2.30^{b}	0.70	0.31^{b}	0.42^{a}	0.39^{b}	3.66	5.35^{b}
Trifolium glomeratum L.							
CFA	10.66^{a}	1.99	0.50^{a}	0.66^{b}	0.63^{a}	5.63	6.72^{a}
NFA	10.68^{a}	2.01	0.49^{a}	0.65^{ab}	0.62^{ab}	5.56	6.66^{a}
NFA+	8.65^{b}	1.35	0.41^{b}	0.62^{a}	0.57^{b}	7.53	9.97^{b}
Trifolium striatum L.							
CFA	6.87^{a}	1.85^{a}	0.65^{a}	0.71^{b}	0.69^{a}	3.75	3.42^{a}
NFA	4.92^{b}	1.39^{ab}	0.60^{b}	0.67^{b}	0.66^{a}	3.71	4.11^{ab}
NFA+	3.36^{b}	0.95^{b}	0.49^{c}	0.57^{a}	0.55^{b}	3.63	4.55^{b}
Trifolium subterraneum L.							
CFA	13.79	2.70^{a}	0.27^{a}	0.37^{b}	0.35^{a}	5.20	6.68^{a}
NFA	13.17	1.83^{b}	0.23^{b}	0.39^{b}	0.36^{a}	7.53	9.28^{ab}
NFA+	9.98	1.34^{b}	0.17^{c}	0.34^{a}	0.29^{b}	7.58	10.75^{b}

Different letters indicate significant effects between O_3 treatments ($p < 0.05$). CFA, charcoal filtered air; NFA, non-filtered air, NFA+, non-filtered air supplemented with 40 ppb of O_3.

Table 3
Plant growth-related parameters of the grasses grown in the different O_3 treatments

	Root RGR	Aerial RGR	Total RGR	Shoot/root
Aegilops geniculata Roth				
CFA	0.35^{a}	0.35	0.36	1.53
NFA	0.34^{a}	0.34	0.33	1.19
NFA+	0.27^{b}	0.31	0.29	1.72
Aegilops triuncialis L.				
CFA	0.37	0.41	0.38	1.59
NFA	0.39	0.42	0.40	1.66
NFA+	0.37	0.42	0.39	1.90
Avena sterilis L.				
CFA	0.61	0.57	0.59	0.25
NFA	0.61	0.57	0.59	0.22
NFA+	0.59	0.55	0.58	0.41
Briza maxima L.				
CFA	0.61	0.59	0.60	2.49
NFA	0.66	0.60	0.63	1.85
NFA+	0.61	0.57	0.59	2.71
Bromus hordeaceus L.				
CFA	0.60	0.48	0.53	0.63
NFA	0.58	0.49	0.54	0.64
NFA+	0.52	0.48	0.50	1.06
Bromus sterilis L.				
CFA	–	0.53	–	–
NFA	–	0.52	–	–
NFA+	–	0.51	–	–
Lolium rigidum Gaudin				
CFA	0.83	0.72	0.75	1.56
NFA	0.83	0.69	0.73	1.33
NFA+	0.81	0.68	0.72	1.51
Cynosurus echinatus L.				
CFA	0.62^{a}	0.73	0.69^{a}	2.98^{a}
NFA	0.70^{b}	0.74	0.73^{b}	1.12^{b}
NFA+	0.65^{ab}	0.74	0.70^{a}	2.26^{a}
Vulpia myuros (L.) C. C. Gmelin				
CFA	–	0.55	–	–
NFA	–	0.52	–	–
NFA+	–	0.55	–	–

Different letters indicate significant effects between O_3 treatments ($p < 0.05$). CFA, charcoal filtered air; NFA, non-filtered air, NFA+, non-filtered air supplemented with 40 ppb of O_3.

and 67%, respectively, of the assessed Leguminosae species and caused a reduction in the total RGR of 78% of the species of this family. The root RGR was only affected in 29% of the Gramineae species involved in the experiment, while no detrimental effects were found on their aerial biomass RGR and a single species of this family showed a significant effect on its total RGR.

A combined analysis using a two-way ANOVA was carried out to assess whether total RGR was affected by ozone exposure and/or plant family (see Fig. 1). Ozone significantly ($p < 0.05$) affected RGRs, inducing lower rates in the NFA+ treatment when compared to CFA or NFA plants. Similarly, grass species significantly ($p < 0.0001$) presented greater RGRs than legumes while a trend ($p = 0.07$) towards a family-O_3 interaction was found. Grasses and legumes presented similar RGRs in the CFA treatment while the total RGR of grasses was greater than for legumes when both taxa were exposed to the NFA or NFA+ treatments. This pattern of sensitivity for both plant families based on growth responses to O_3 is in agreement with that found when foliar visible injury was considered (Bermejo et al., 2003), although in this experiment a significant O_3–family interaction was detected. These results match with other studies reporting a greater sensitivity of the Leguminosae family when compared with Gramineae species (Bungener et al., 1999; Warwick and Taylor, 1995). Therefore a separate analysis of the results was carried out for both families.

3.1. Ozone effects on species of the Leguminosae family

Ambient O_3 levels (NF treatment) corresponding to AOT40 values ranging 92–775 ppb h (Table 1) caused significant reductions in the aerial biomass (foliage or stem biomass) of *T. striatum*, *T. subterraneum* and *Medicago minima* (Table 2). Ozone affected both the foliage and the stem of *Medicago,* that showed reductions of 41 and 36%, respectively, when compared with the control treatment. A similar pattern was found in *T. striatum*, however, ambient O_3 levels only significantly affected its foliage dry weight (by 28%), while shoot biomass was the most affected parameter in *T. subterraneum* (32%). Ambient O_3 exposure also induced adverse effects on root RGR of *T. striatum* and *T. subterraneum*, with reductions ranging 8–15% when compared with control plants.

Above ambient O_3 levels (NF+ treatment) corresponding to AOT40 values ranging 7222–15,493 ppb h (Table 1) induced detrimental effects in the range of 6–14% in the aerial RGR of *T. angustifolium*, *Trifolium glomeratum* and *T. subterraneum* and of ca. 20% in *Biserrula pelecinus*, *M. minima*, *Trifolium cherleri* and *T. striatum*. These O_3 levels determined reductions (147–37%) in the root RGR of these species except for *Biserrula*, where no effects were detected. The total RGR was adversely affected in these seven species following their exposure to the NFA+ treatment, reductions in the 10–21% range were found when compared with control plants, *Medicago* and *T. striatum* being the most affected species. The intensity of the O_3-induced adverse effects are in the range of those reported by Warwick and Taylor (1995) regarding the sensitivity of calcareous herbaceous species, where the most sensitive species showed 20% depletions in its root and shoot RGRs. However, in our study the greatest adverse effect was found in the root RGR of *T. subterraneum* (37%). Ozone exposure did not determine any effect on the root, aerial or total RGR of *Anthyllis cornicina*, *Anthyllis lotoides* and *O. compressus*.

Table 4
Ozone-induced effects on plant canopy-related parameters of the dehesa therophytes involved in the experiment

Exposure	*Leguminosae*				*Gramineae*			
	Diameter (cm)	Height (cm)	Surface (m^2)	Vol. (dm^3)	Diameter (cm)	Height (cm)	Surface (m^2)	Vol. (dm^3)
	Anthyllis cornicina L.				*Aegilops geniculata* Roth			
CFA	26.66	7.16	0.072	5.32	46.53	14.18^{a}	0.22	31
NFA	26.08	7.50	0.069	5.40	46.46	15.92^{a}	0.22	35
NFA+	23.91	7.17	0.058	4.13	47.78	12.00^{b}	0.23	29
	Anthyllis lotoides L.				*Aegilops triuncialis*			
CFA	48.88	8.44^{a}	0.24	21.26	57.03	14.43	0.33	48
NFA	49.5	8.77^{a}	0.25	22.77	59.08	14.16	0.37	51
NFA+	45.33	7.33^{b}	0.23	16.49	57.96	14.07	0.35	48
	Biserrula pelecinus L.				*Avena sterilis* L.			
CFA	62.3^{a}	6.6	0.39^{a}	25.72^{a}	103.29^{a}	18.91	1.08	199^{a}
NFA	59.2^{a}	–	0.37^{a}	–	100.54^{a}	17.5	1.02	174^{a}
NFA+	40.4^{b}	6.2	0.17^{b}	10.63^{b}	87.16^{b}	16.92	0.77	128^{b}
	Medicago minima (L.) Bartal				*Briza maxima* L.			
CFA	19.04^{a}	4.41^{a}	0.037^{a}	1.66^{a}	55.13	19.81	0.31	61^{a}
NFA	17.66^{ab}	3.91^{ab}	0.032	1.31^{ab}	56.83	20.08	0.33	68^{a}
NFA+	16.25^{b}	3.33^{b}	0.027^{b}	0.91^{b}	49.29	18.00	0.25	45^{b}
	Ornithopus compressus L.				*Bromus hordeaceus* L.			
CFA	38.44^{a}	8.00	0.15^{a}	12.07	67.95	34.08^{a}	0.46	161
NFA	32.92^{b}	8.28	0.11^{b}	9.17	66.87	28.25^{b}	0.46	132
NFA+	35.00^{b}	8.41	0.12^{b}	11.04	64.16	28.00^{b}	0.42	119
	Trifolium angustifolium L.				*Bromus sterilis* L.			
CFA	28.09^{b}	12.18	0.079^{a}	9.71^{a}	61.83	24.44^{a}	0.38	94
NFA	25.62^{b}	11.83	0.066^{b}	7.94^{a}	63.36	22.42^{b}	0.40	89
NFA+	22.20^{a}	10.58	0.049^{c}	5.52b	64.33	23.00^{b}	0.42	96
	Trifolium cherleri L.				*Lolium rigidum* Gaudin			
CFA	20.46	8.33	0.046	3.44	65.66	19.88	0.44	87
NFA	19.53	6.50	0.040	2.69	68.05	20.66	0.47	98
NFA+	18.54	7.33	0.036	2.37	71.25	20.13	0.52	106
	Trifolium glomeratum L.				*Cynosurus echinatus* L.			
CFA	30.60	7.38^{a}	0.13	9.76	49.5^{a}	25.55^{a}	0.24	62^{a}
NFA	34.71	6.07^{b}	0.12	7.57	50.38^{a}	22.66^{b}	0.27	61^{ab}
NFA+	28.80	5.69^{b}	0.11	7.08	46.56^{b}	23.44^{b}	0.22	51^{b}
	Trifolium striatum L.				*Vulpia myuros* (L.) C. C. Gmelin			
CFA	30.10	12.90^{a}	0.090^{a}	11.72^{a}	60.58^{a}	27.5^{a}	0.37	102^{a}
NFA	26.30	11.10^{b}	0.069^{b}	7.80^{b}	54.77^{b}	22.72^{b}	0.30	71^{b}
NFA+	22.13	9.92^{b}	0.050^{c}	5.22^{c}	57.25	24.70^{ab}	0.33	81^{ab}
	Trifolium subterraneum L.							
CFA	38.12^{a}	14.00^{a}	0.150^{a}	22.10^{a}				
NFA	31.08^{b}	10.66^{b}	0.100^{a}	11.50^{b}				
NFA+	29.41^{b}	11.08^{b}	0.088^{b}	10.13^{b}				

Different letters indicate significant effects between O_3 treatments ($p < 0.05$). CFA, charcoal filtered air; NFA, non-filtered air, NFA+, non-filtered air supplemented with 40 ppb of O_3.

Ozone exposure determined shifts in the shoot/root dry weight biomass ratio of 55% of the legumes involved in the experiment. Ambient (AOT40 = 783 ppb h) and above ambient O_3 levels (AOT40 = 15,493 ppb h) induced average reductions of 33% in the shoot/root ratio of *T. cherleri*, when compared to CFA plants. Similarly, the *O. compressus* plants grown in the NFA+ chambers presented a 29% lower shoot/root ratio than CFA plants, in association with AOT40 values of 12,099 ppb h. On the contrary, increases in the shoot/root ratio, influenced by a greater O_3 adverse effect on the root systems, were found in the NFA+ plants (AOT40 values over 11400 ppb h) of *T. subterraneum*, *T. striatum* and *T. glomeratum* (33–60%) when compared with those grown in the CFA treatment. The largest O_3-induced increase in the shoot/root ratio was found in *T. subterraneum* (38%). The alteration of carbon allocation is a common effect induced by O_3 (Cooley and Manning, 1987) and has also been reported in similar experiments involving the exposure of herbaceous

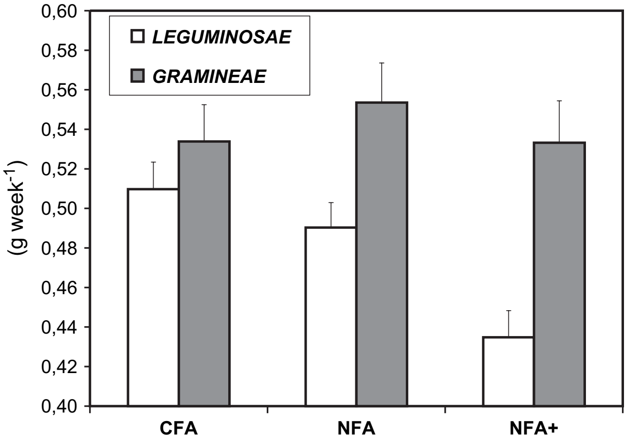

Fig. 1. Average total relative growth rate for Leguminosae (○) and Gramineae (●) plants grown in the different O_3 treatments. CFA = charcoal filtered air; NFA = non-filtered air; NFA+ = non-filtered air supplemented with 40 ppb O_3. Mean ± SE.

species. However, shoot or root partitioning was species-dependent (Franzaring et al., 2000; Reiling and Davison, 1992; Warwick and Taylor, 1995).

The observed changes in shoot/root dry weight biomass ratio or in the root RGR might determine important ecological implications in the dehesa Mediterranean systems that usually experience water and nutrient limitations. A significant amount of the photosynthates of these species is usually accumulated in the root system to overcome stressful periods when soil moisture and nutrient availability are limited. Therefore the observed shifts induced by O_3 on shoot/root ratio may affect the competitive ability of these species (Aers et al., 1991; Franzaring et al., 2000).

Changes in plant morphology were observed in all the species following their exposure to O_3 except for *A. cornicina* and *T. cherleri* (see Table 4). These effects did not necessarily correspond with the observed responses in aerial or subterranean biomass or RGR, as was the case for *O. compressus*.

A ranking of decreasing O_3 sensitivity is proposed for the Leguminosae species involved in the experiment, based on the changes induced by ambient and above ambient levels of this pollutant on the aerial, root and total RGRs and the shoot/root ratio. The responses of the morphological parameters were used to fine-tune the O_3 sensitivity of the different species. The first criterion was to select those species showing adverse effects on their RGRs following their exposure to ambient O_3 levels (*T. striatum*, *T. subterraneum*). The second criterion was to choose those species where above ambient O_3 levels induced detrimental effects on their RGRs and were simultaneously affected by ambient levels on morphological parameters (*T. cherleri*, *T. angustifolium*). A third sensitivity group was defined by those species that only showed adverse effects when exposed to the NF+ treatment (*M. minima*, *T. glomeratum*, *Biserrula*). The group encompassing the least sensitive species was defined by the lack of response of their RGR even when exposed to the maximum O_3 levels or just showing adverse effects on a single morphological parameter (the two *Anthyllis* species). *O. compressus* was considered slightly more sensitive than this group since O_3 exposure altered its shoot/root ratio. As a result the following ranking is proposed:

> *T. striatum*, *T. subterraneum* > *T. cherleri*, *T. angustifolium* > *M. minima*, *T. glomeratum*, *B. pelecinus* > *O. compressus* > *A. cornicina*, *A. lotoides*.

This sensitivity ranking based on growth-related parameters showed a great similarity with that based on the induction of foliar injury reported by Bermejo et al. (2003). However, *T. glomeratum* was more resistant than expected from visible injury responses while the opposite was true for *M. minima*. These types of disagreements in the sensitivity classifications when visible injury or growth-related parameters have been reported by many authors in the literature (see review by Davison and Barnes, 1998). It is worth noting the high sensitivity of the *Trifolium* species involved in the study, in agreement with Nebel and Fuhrer (1994), Nussbaum et al. (1995), Karlsson et al. (1995), Balls et al. (1996), Ashmore et al. (1996), Bergmann et al. (1999) and Gimeno et al. (2004). Since the ecology of these *Trifolium* species is quite different, Bergmann et al. (1999) suggested that their great sensitivity could be related to evolutionary and genetic features.

3.2. Ozone effects on species of the Gramineae family

Significant O_3 effects on the aerial, root or total RGRs were found in only two of the Gramineae species involved in the experiment (see Table 3). Above ambient O_3 levels (AOT40 = 14,183 ppb h) caused a 23% reduction in the root RGR of *Aegilops geniculata*. Ambient O_3 levels (AOT40 = 415 ppb h) induced a 13% increase in this parameter on *Cynosurus echinatus* when compared with CFA plants. The aerial RGR was not affected on any of the assessed grass species. Ambient O_3 (415 ppb h) levels induced a 6% increase in the total biomass RGR of *Cynosurus echinatus*; no effects on this parameter were found for the remaining grass species.

Regarding O_3 effects on plant structure and canopy architecture (see Tables 3 and 4), ambient O_3 levels determined a reduction in the shoot/root ratio of *C. echinatus*. No effects on this parameter were found in any of the remaining species. Ambient O_3 levels with AOT40 values ranging from 327–818 ppb h induced 87–17% reductions in the height of *Bromus hordeaceus*, *B. sterilis*, *C. echinatus* and *Vulpia myuros*. Above ambient O_3 levels (AOT40 levels in the 12,500–14,200 ppb h range) determined 6–18% reductions in the height of *A. geniculata*, *B. hordeaceus*, *B. sterilis* and *C. echinatus*. No effects on plant surface were detected in any grass species when exposed to the NFA or NFA+ treatment. A 21% reduction was induced by ambient O_3 exposure (AOT40 = 327 ppb h) in the plant canopy volume of *V. myuros* plants, when compared to CFA-treated plants. Above ambient O_3 levels corresponding to AOT40 values in the 8500–12,800 ppb h range were associated with reductions of 36, 26 and 18% in the plant volume of *A. sterilis*, *B. maxima* and *C. echinatus*, respectively.

Although the grass species involved in the experiment were rather insensitive to O_3, they were ranked according to the AOT40 levels that determined effects on biomass RGR and alterations in plant morphology. The first criterion followed to classify these species was the detection of an effect on any RGR (*Cynosurus echinatus* and *Aegylops geniculata*). *Cynosurus echinatus* was classified as a sensitive species although O_3 exposure induced increases and reductions in some of the assessed parameters. The second criterion adopted was that any alteration in plant performance would be indicative of plant sensitivity. Three groups were defined according to this criterion. The first group involved those species that showed alterations in morphological parameters when exposed to ambient O_3 levels (*V. myuros*, *B. hordeaceus* and *B. sterilis*). The second group encompassed those species showing morphological changes following their exposure to above ambient O_3 levels (*Avena sterilis* and *B. maxima*). Ozone exposure did not induce effects on any of the assessed parameters of *A. triuncialis* and *L. rigidum*, thus forming the least sensitive group. As a result, the Gramineae species are ranked as follows:

> *C. echinatus*, *A. geniculata* > *V. myuros*, *B. hordeaceus*, *B. sterilis* > *Avena sterilis*, *B. maxima* > *A. Triuncialis*, *L. rigidum*

As was the case for legumes, discrepancies were found between this sensitivity ranking and that proposed in Bermejo et al. (2003) for the same species based on foliar injury. The most remarkable disagreement is observed in *C. echinatus* and *V. myuros*, that would be most sensitive according to growth but insensitive regarding visible injury.

Some authors have attempted to evaluate whether the C-SR model of plant strategies defined by Grime (1979) could be associated with O_3 susceptibility. Reiling and Davison (1992), Selldén and Pleijel (1995), Bungener et al. (1999) and Franzaring et al. (2000) have found that the most sensitive species of their studies presented a high component of C or R strategies (R/CR or CR) and were the fastest-growing species, showing the greatest RGR in clean air. However, in our experiment plant sensitivity to O_3 was neither explained by its RGR values in clean air (Table 2 and 3) nor by its CSR classification (data not shown), in agreement with the reports from other authors (Pleijel and Danielsson, 1997; Warwick and Taylor, 1995) that did not find a clear association between these plant traits and O_3 sensitivity.

The AOT40 was the exposure index that best explained the observed effects of O_3 on the aerial, subterranean or total RGR of grasses and legumes of our experiment, when compared with other indices based on mean, maximum values or accumulated indices using other cut-offs (data not shown). AOT40 was selected to derive equations relating the aerial, subterranean or total RGR of grasses and legumes to O_3 exposure (see Fig. 2). In these equations the observed responses were expressed relative to the CFA treatment (100% growth). Because Leguminosae and Gramineae species showed a great disparity regarding their O_3 sensitivity, separate equations were also carried for both taxa. According to the suggestions provided by Ashmore and Franzaring (2003) the two most resistant Leguminosae species were not included in the analysis

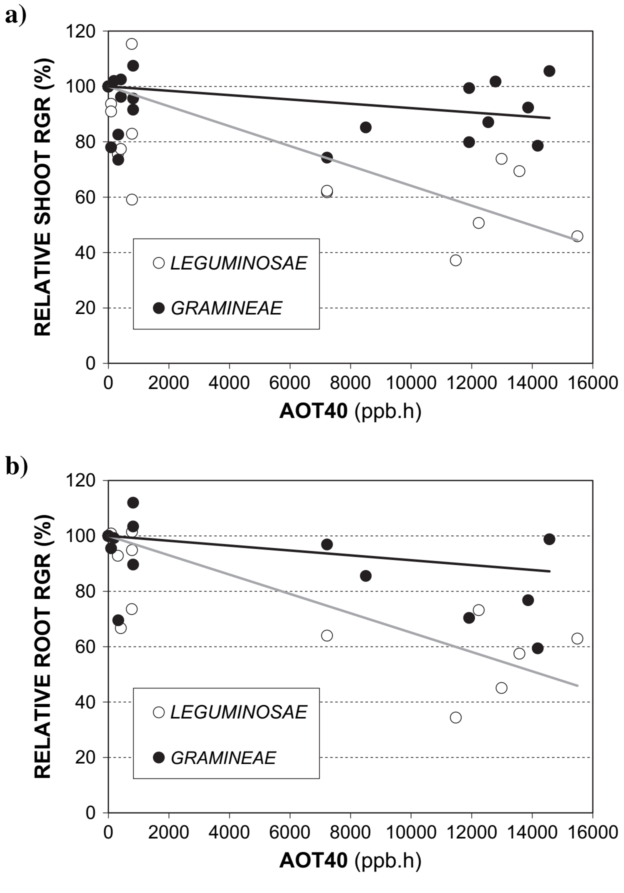

Fig. 2. Growth response of the plants involved in the experiment in relation to ozone exposure (AOT40, ppb h). Models were constructed for Leguminosae plants (lighter line) and Gramineae plants (darker line). (a) Relative shoot RGR; Gramineae: $y = -0.0008x + 100$ ($r^2 = -0.08$, ns); Leguminosae: $y = -0.0036x + 100$ ($r^2 = 0.51$, $p < 0.01$). (b) Relative root RGR; Gramineae $y = -0.0009x + 100$ ($r^2 = 0.02$, ns); Leguminosae: $y = -0.0035x + 100$ ($r^2 = 0.64$, $p < 0.01$).

(see Fig. 2). The best fit was found for the subterranean biomass RGR of legumes plants ($r^2 = 0.64$), followed by the total and aerial biomass RGR of this group (r^2 values of 0.57 and 0.51, respectively). Non-significant relationships between O_3 exposure and the performance of Gramineae species were found. Ten per cent reductions in the RGRs of the Leguminosae plants would be expected following exposures to AOT40 values close to 2800 ppb accumulated in a range of 43–67days, in agreement with present critical level for the protection of semi-natural grasslands, 3000 ppb h over a period of up to three months.

In summary, the legumes involved in this screening experiment were more sensitive to O_3 than the grass species. Ozone caused detrimental effects on the aerial, subterranean or total biomass of most of the Leguminosae species. Changes in plant morphology were also observed in many legumes following their exposure to O_3 levels. Further studies will be carried out to evaluate whether these changes could affect plant O_3 uptake. The O_3 exposure that induced detrimental effects on the most sensitive species was in the range of the current critical level (Fuhrer et al., 2003). The results of this study indicate that O_3 exposure might induce changes in the composition of dehesa grasslands by decreasing the performance of legumes when compared to grasses, and thus affecting the structure and function of these ecosystems and their forage quality. More research is needed to assess the impact of O_3 on the competitive ability of these species and its interactive effects with nitrogen and water availability.

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References

Alonso, R., Elvira, S., Castillo, F.J., Gimeno, B.S., 2001. Interactive effects of ozone and drought stress on pigments and activities of oxidative enzymes in *Pinus halepensis*. Plant Cell and Environment 24, 905–916.

Aers, R., Boot, R.G.A., van der Aart, P.J.M., 1991. The relation between above- and below-ground biomass allocation patterns and competitive ability. Oecologia 87, 551–559.

Ashmore, M.R., Ainsworth, N., 1995. The effects of ozone and cutting on the species composition of artificial grassland communities. Functional Ecology 9, 708–712.

Ashmore, M.R., Franzaring, J., 2003. Report from the working group of semi-natural vegetation. In: Karsslson, P.E., Sellden, G., Pleijel, H. (Eds.), Establishing ozone critical levels II. UNECE Workshop Report. IVL Swedish Environmental Institute, University of Göteborg, pp. 23–27.

Ashmore, M.R., Power, S.A., Cousins, D.A., Ainsworth, N., 2003. Effects of ozone on native grass and legumes species: a comparison of responses of individual plants and artificial communities. In: Kärenlämpi, L., Skarby, L. (Eds.), Critical levels for ozone in Europe. UNECE Workshop report. University of Kuopio, Dept. of Ecology and Environmental Science, pp. 104–108.

Azcárate, F.M., Sánchez, A.M., Arqueros, L., Peco, B., 2002. Abundance and habitat segregation in Mediterranean grassland species: the importance of seed weight. Journal of Vegetation Science 13, 159–166.

Balls, G.R., Palmer-Brown, D., Sanders, G.E., 1996. Investigating microclimatic influences on ozone injury on clover (*Trifolium subterraneum*) using artificial neural networks. New Phytologist 132, 271–280.

Barbo, D.N., Chappelka, A.H., Somers, G.L., Miller-Goodman, M.S., Stolke, K.W., 1998. Diversity of an early successional plant community as influenced by ozone. New Phytologist 138, 291–298.

Bergmann, E., Bender, J., Weigel, H.-J., 1999. Ozone threshold doses and exposure–response relationships for the development of ozone injury symptoms in wild plant species. New Phytologist 144, 423–435.

Bermejo, V., Gimeno, B.S., Sanz, J., de la Torre, D., Gil, J.M., 2003. Assessment of ozone sensitivity of 22 native plant species from Mediterranean annual pastures based on visible injury. Atmospheric Environment 37, 4667–4677.

Bungener, P., Balls, G.R., Nussbaum, S., Geissmann, M., Grub, A., Fuhrer, J., 1999. Leaf injury characteristics of grassland species exposed to ozone in relation to soil moisture condition and vapour pressure deficit. New Phytologist 142, 271–282.

Cooley, D.R., Manning, W.J., 1987. The impact of ozone on assimilate partitioning in plants: a review. Environmental Pollution 47, 95–113.

Davison, A.W., Barnes, J.D., 1998. Effects of ozone on wild plants. New Phytologist 139, 135–151.

El-Khatib, A.A., 2003. The response of some common Egyptian plants to ozone and their use as bioindicators. Environmental Pollution 124, 419–428.

EMEP, 2002. Transboundary acidification, eutrophication and ground level ozone in Europe. EMEP Report 1 and 2/2002. Oslo, Norway, EMEP.

Franzaring, J., Tonniejck, A.E.G., Koijman, A.W.N., Dueck, Th.A., 2000. Growth responses to ozone in plant species from wetlands. Environmental and Experimental Botany 44, 39–48.

Fuhrer, J., Ashmore, M.R., Mills, G., Hayes, F., Davison, A.W., 2003. Ozone critical levels for semi-natural vegetation. In: Karlsson, P.E., Sellden, G., Pleijel, H. (Eds.), Establishing ozone critical levels II. UNECE Workshop Report. IVL Swedish Environmental Institute, Göteborg, Sweden, pp. 183–198.

Fuhrer, J., Shariat-Madari, H., Perler, R., Tschannen, W., Grub, A., 1994. Effects of ozone on managed pasture. II. Yield, species composition, canopy structure and forage quality. Environmental Pollution 83, 317–325.

Gimeno, B.S., Bermejo, V., Reinert, R.A., Zheng, Y., Barnes, J.D., 1999. Adverse effects of ambient ozone on watermelon yield and physiology at a rural site in Eastern Spain. New Phytologist 144, 245–260.

Gimeno, B.S., Bermejo, V., Sanz, J., de la Torre, D., Gil, J.M., 2004. Assessment of the effects of ozone exposure and plant competition on the reproductive ability of three therophytic clover species from Iberian pastures. Atmospheric Environment, 2295–2303.

Grime, J.P., 1979. Plant strategies and vegetation processes. Wiley, Chichester.

Hunt, R., 1990. Basic growth analysis. Unwin Hyman, London, U.K.

Karlsson, G., Pleijel, H., Sild, E., Danielsson, H., Selldén, G., Ericson, L., Skärby, L., et al., 1995. Clover Sweden—a national three year study of the effects of tropospheric ozone on *Trifolium subterraneum*. Water, Air and Soil Pollution 85, 1503–1508.

Madkour, S.A., Laurence, J.A., 2002. Egyptian plant species as new ozone indicators. Environmental Pollution 120, 339–353.

Milliken, G.A., Johnson, D.E., 1992. Designed experiments. Analysis of messy data, vol. I. Chapman & Hall, New York, USA.

Montoya, J.M., Mesón, M.L., Ruiz del Castillo, J., 1988. Una dehesa testigo. La dehesa de Moncalvillo. Serie técnica n° 5. Ministerio de Agricultura Pesca y Alimentación, Madrid, Spain.

Nebel, B., Fuhrer, J., 1994. Inter and intra-specific differences in ozone sensitivity in semi-natural plant communities. Angewandte Botanik 68, 116–121.

Nussbaum, S., Geissmann, M., Fuhrer, J., 1995. Ozone exposure relationships for mixtures of perennial ryegrass and white clover depend on ozone exposure patterns. Atmospheric Environment 29, 989–995.

Palacios, M., Kirchner, F., Martilli, A., Clappier, A., Martín, F., Rodríguez, M.E., 2002. Summer ozone episodes in the Greater Madrid area. Analysing the ozone response to abatement strategies by modelling. Atmospheric Environment 36, 5323–5333.

Pineda, F.D., de Miguel, J.M., Casado, M.A., Montalvo, J., 2002. Claves para comprender la diversidad biológica y conservar la biodiversidad. In: Pineda, F.D., de Miguel, J.M., Casado, M.A., Montalvo, J. (Eds.), La diversidad biológica en España. Prentice Hall, Madrid, Spain, pp. 7–30.

Plaza, J., Pujadas, M., Artíñano, B., 1997. Formation and transport of the Madrid ozone plume. Journal of Air and Waste Management Association 47, 666–774.

Pleijel, H., Danielsson, H., 1997. Growth of 27 herbs and grasses in relation to ozone exposure and plant strategy. New Phytologist 135, 361–367.

Reiling, K., Davison, A.W., 1992. Effects of a short ozone exposure given at different stages in the development of *Plantago major* L. New Phytologist 121, 643–647.

San Miguel, A., 1994. La dehesa española. origen, tipología, características y gestión. E.T.S.I.M, Madrid, Spain.

Selldén, G., Pleijel, H., 1995. Photochemical oxidant effects on vegetation-response in relation to plant strategy. Water Air and Soil Pollution 85, 111–122.

Velissariou, D., Davison, A.W., 1994. Ozone effects on important Greek fodder crops. In: Critical levels for ozone. Fuhrer, J., Achermann, B. (Eds.), A UNECE workshop report, Schriftenreihe der FAC No 16, Berne-Liebefeld, pp. 252–255.

Warwick, K., Taylor, R.G., 1995. Contrasting effects of tropospheric ozone on five native herbs which coexist in calcareous grassland. Global Change Biology 1, 143–151.

3.2. EFECTOS DEL OZONO EN LA CAPACIDAD REPRODUCTIVA DE TRES TRÉBOLES MEDITERRÁNEOS ANUALES CONSIDERANDO EL FACTOR COMPETENCIA

Se analiza el efecto del O_3 sobre la capacidad reproductiva de tres tréboles anuales mediterráneos considerando el factor competencia: *Trifolium cherleri*, *Trifoliun striatum* y *Trifolium subterraneum*. Los experimentos se desarrollaron en una instalación de cámaras de techo descubierto (OTCs). Se consideraron tres tratamientos de O_3: aire filtrado, cuya concentración no sobrepasa las 15 ppb (CFA); aire no filtrado, que reproduce las concentraciones ambientales del contaminante (NFA); y aire no filtrado al que se le añaden 40 ppb de O_3 por encima del ambiente (NFA+). Se utilizó la gramínea *Briza máxima*, una especie con mayor tolerancia al O_3 como especie competidora. Se diseñaron tres mesocosmos: monocultivos de cuatro plantas de trébol, tres plantas de trébol compitiendo con una planta de *Briza máxima* y una planta de trébol compitiendo con tres plantas de *Briza máxima*.

Figura 11. Mesocosmos compuestos con especies herbáceas anuales en el interior de una OTC (Autor: J. Sanz).

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Assessment of the effects of ozone exposure and plant competition on the reproductive ability of three therophytic clover species from Iberian pastures

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Abstract

Ozone (O_3) phytototoxicity has been reported on a wide range of crops and wild Central European plant species, however no information has been provided regarding the sensitivity of plant species from dehesa Mediterranean therophytic grasslands in spite of their great plant species richness and the high O_3 levels that are recorded in this area. A study was carried out in open-top chambers (OTCs) to assess the effects of O_3 and competition on the reproductive ability of three clover species: *Trifolium cherleri*, *Trifolium subterraneum* and *Trifolium striatum*. A phytometer approach was followed, therefore plants of these species were grown in mesoscosms composed of monocultures of four plants of each species, of three plants of each species competing against a *Briza maxima* individual or of a single plant of each clover species competing with three *B. maxima* plants. Three O_3 treatments were adopted: charcoal filtered air (CFA), non-filtered air (NFA) and non-filtered air supplemented with $40\,\mathrm{nl\,l^{-1}}$ of O_3 (NFA+). The different mesocosms were exposed to the different O_3 treatments for 45 days and then they remained in the open. Ozone exposure caused reductions in the flower biomass of the three clover species assessed. In the case of *T. cherleri* and *T. subterraneum* this effect was found following their exposure to the different O_3 treatments during their vegetative period. An attenuation of these effects was found when the plants remained in the open. Ozone-induced detrimental effects on the seed output of *T. striatum* were also observed. The flower biomass of the clover plants grown in monocultures was greater than when competing with one or three *B. maxima* individuals. An increased flower biomass was found in the CFA monoculture mesocosms of *T. cherleri* when compared with the remaining mesocosms, once the plants were exposed in the open for 60 days. The implications of these effects on the performance of dehesa acid grasslands and for the definition of O_3 critical levels is discussed.

Keywords: Seed production; Flower biomass; Mediterranean grasslands; Dehesa; Critical levels

1. Introduction

Grassland ecosystems are particularly endangered by the interaction of global change components such as alterations in land-use changes, climate, nitrogen deposition, biotic exchange or atmospheric carbon dioxide (Sala et al., 2000). Tropospheric ozone (O_3) is also a global change compound that might harm these ecosystems at the European scale since it reaches phytotoxic levels in rural areas (EMEP, 2002). Ozone-induced detrimental effects on grassland species from Northern and Central Europe have been reported, involving the induction of visible injury, reductions in growth-related parameters or alterations in plant competitive ability (Davison and Barnes, 1998). However, there is scarce information regarding O_3 impacts on Mediterranean grasslands although they present a

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remarkable plant species richness, as is the case for dehesa-type grasslands (Pineda et al., 1981), and the Mediterranean basin has been considered as an "hyper-hot" candidate for conservation support (Myers et al., 2000).

There is evidence that high O_3 levels are reached in the dehesas of the central Iberian rangelands (Plaza et al., 1997; Palacios et al., 2002). These ecosystems are agro-sylvo-pastoral systems composed of cleared oak woodlands with an annual grassland understorey and represent an example of sustainable management of natural resources in the Iberian Peninsula, covering agricultural, timber and extensive livestock exploitation (Marañón, 1988). Large spatial and temporal fluctuations in their floristic composition occur depending on tree coverage, slope-valley gradients, grazing pressure, regular ploughing and meteorological fluctuations (Peco et al., 1998a).

The most characteristic herbaceous species of therophytic dehesa grasslands on acid soils belong to the *Leguminosae* or *Graminae* families; both taxa have different roles in ecosystem function, mainly in nitrogen cycling, and they also have dissimilar nutritive implications for the livestock grazing on them. A screening assessment based on visible injury indicated that dehesa clover species, such as *Trifolium cheleri*, *Trifolium striatum* and *Trifolium subterraneum*, were more sensitive to O_3 than the grasses of these ecosystems (Bermejo et al., 2003). These findings are in agreement with the reports provided by other authors in regards to the sensitivity of the *Trifolium* genus to O_3 (Nussbaum et al., 1995; Warwick and Taylor, 1995; Bungener et al., 1999). However, while the ecological significance of visible symptoms and reduced biomass is unknown, potential O_3 effects on plant reproductive ability (see review by Black et al., 2000) are of undoubted ecological relevance (Davison and Barnes, 1998). This is specially valid for Mediterranean rangelands dominated by therophytes as a great correspondence between the soil seed bank and the plant species present in these ecosystems has been found (Peco et al., 1998b). Moreover, it is difficult to scale-up these results to plant community level since interactions of this pollutant with other abiotic and biotic factors are likely to occur. Experiments involving plant competition are needed to predict dynamic changes in plant communities under O_3 stress as this pollutant has been reported to induce changes in the species composition of grassland communities (Fuhrer et al., 1994; Ashmore et al., 1995). Also, O_3 exposure pattern might influence plant responsiveness as carry-over effects of this pollutant were found when therophytes from dehesa grasslands were exposed during the earlier stages of their short life- span (Bermejo et al., 2003).

The United Nations—Economic Commission for Europe Convention on Long-Range Transboundary Air Pollution (UN ECE CLRTAP) has promoted the establishment of O_3 critical levels for plant receptors. Critical level is defined as the pollutant concentration above which direct effects on receptors such as plants, ecosystems or materials, may occur according to present knowledge (UN ECE, 1988). The definition of O_3 critical levels for seminatural vegetation is a difficult matter because of the great diversity of these receptors, the complexity of interactions between O_3 effects and environmental conditions and also due to the lack of experimental data (Fuhrer et al., 2003). Sensitive taxa and ecosystems should be identified to establish sound critical levels for seminatural vegetation (Davison and Barnes, 1998). Present O_3 critical level for this receptor is established as an AOT40 (Accumulated exposure over $40\,\mathrm{nl\,l^{-1}\,h}$) value of $3000\,\mathrm{nl\,l^{-1}}$ h for three consecutive months, although a great uncertainty in this estimate was found regarding both the exposure index and its value (Ashmore and Franzaring, 2003).

The aims of this work were to determine whether an early O_3 exposure would induce detrimental effects in the reproductive ability of three clover therophytes from dehesa acidic grasslands and to ascertain whether plant competition modulates this impact. An analysis is also made to find the most suitable O_3 exposure index best related with the response of these species aiming to contribute to the definition of O_3 critical levels since an urgent need in providing information on the response of Mediterranean species to O_3 has been identified (Ashmore and Franzaring, 2003).

2. Materials and methods

2.1. Plant material

Three O_3-sensitive clover species, *T. cherleri*, *T. subterraneum* and *T. striatum*, from dehesa acidic grasslands were grown in monoculture or in competition with an O_3-resistant grass species from the same ecosystems (*Briza maxima*). The O_3 sensitivity of these species was assessed in a former experiment (Bermejo et al., 2003). The germoplasm bank of the *Consejería de Agricultura y Medio Ambiente de la Comunidad Autónoma de Extremadura* supplied the clover seeds. The certified seed origin coordinates for *T. cherleri* and *T. striatum* were 38°22′0°64′W, and 38°55′N′ 0°56′W, respectively (Badajoz, Spain). The Spanish *T. subterraneum* cv. Zujar was used in the experiments while *B. maxima* seeds were collected at the Moncalvillo Dehesa in Guadalix de la Sierra (Madrid, 40°40′N 03° 46′W).

The clover seeds of the different species were immersed for 24 h in a Germinator® solution (Agro-Orgánicos Mediterráneos S.L., Spain) to ensure a homogeneous germination. When seeds were swollen they were sown in a medium of 50% neutral peat and

50% vermiculite substrate and grown in a greenhouse. The *B. maxima* seeds did not experience any pre-treatment and were sown using the same substrate. One month after their emergence, plants were transplanted to 6 l pots using a 50% neutral peat, 25% vermiculite and 25% perlite substrate supplemented with 2 kg m^{-3} of a slow-release fertiliser/10–12 months, NPK:12/25/12. The phytometer approach was followed to simulate plant competition in the field aiming to avoid discussion about replacement or additive designs (De Witt, 1960; Snaydon, 1991; Hamilton, 1994). Three mesoscosm types were defined: (a) Four-plant monocultures of *T. cherleri* (A4), *T. striatum* (D4) and *T. subterraneum* (F4); (b) mixtures of 3 *B. maxima* plants growing with a central plant of *T. cherleri* (3B1A), *T. striatum* (3B1D) or *T. subterraneum* (3B1F) and (c) mixtures of 1 *B. maxima* plant growing with 3 plants of *T. cherleri* (3A1B) or *T. striatum* (3D1B). Plants were placed in each pot in the centre and the vertices of a 10 cm side triangle to avoid potential edge-derived effects. Pots were spaced in the chambers avoiding shading effects between plants of different pots. Daily irrigation of plant material was carried out from the start of the experiment using a droplet system to ensure a homogeneous irrigation within each mesocosms.

2.2. Experimental design

The experiment was performed at an open-top chamber (OTC) experimental field located in Sant Jaume d'Enveija, Spain (40°41′N, 0°47′E). When plant height was 4–5 cm and 8–10 cm for clover plants and *B. maxima*, respectively, they were transplanted into pots and transferred into slightly modified NCLAN-type OTCs (Gimeno et al., 1999) to 3 O_3 treatments: charcoal-filtered air (CFA) presenting subphytotoxic O_3 levels, non-filtered air (NFA), and non-filtered air supplemented with 40 nl l^{-1} O_3 from 07:00 to 17:00 (GMT) 5 days week^{-1} (NFA+). Ozone fumigation started a week after plant material was introduced in the chambers, to enable their adaptation to the new environmental conditions. Three chambers per treatment were used following a randomised complete block design. A complete description of the operation of the fumigation and air-quality monitoring systems is provided in Alonso et al. (2001). The different mesocosms were exposed during 45 days to the different O_3 treatments, then the chamber plastics were removed and all plant material remained in the open exposed to ambient O_3 levels. Flower collection was carried out following their formation and maturation, which was both species and time-dependent. Three replicates of each for each mesocosm-type per chamber (9 replicates per each combination of mesocosm and O_3 treatment) were involved in each harvest for each species.

Flowering was first recorded in *T. striatum*, the first evaluation of flower production was carried out once the plant material was already exposed for 45 days in the OTCs. At this stage inflorescences were not developed in *T. subterraneum* and *T. cherleri*. A second assessment of flower dry weight was carried out again in *T. striatum* once the plants remained in the open for 30 days following their previous exposure in the OTCs. This assessment was coincidental with the first evaluation of the flower production of *T. subterraneum* and *T. cherleri*. An additional assessment was carried out for *T. cherleri* once the plants remained in the open for 57 days after their exposure in the OTCs. In each assessment the flowers were carefully excised and dried at 65°C to constant weight.

Table 1
Experimental protocol followed during the experiment

	Parameters	Sowing dates	Start of O_3 treatments	Evaluation date	DAS[a]	AOT40[b] (nl l^{-1} h)		
						CFA	NFA	NFA+
T.cherleri	Flowers	23/04	28/05	8/08	72	1518 (0)	4956 (2662)	17,118 (13,203)
	Flowers	23/04	28/05	8/09	102	2213 (0)	6071 (2662)	18,117 (13,203)
T.striatum	Flowers	23/04	30/05	14/07	45	0 (0)	2404 (2404)	13,390 (13,390)
	Flowers + Seeds	23/04	30/05	13/08	75	1684 (0)	4822 (2404)	16,958 (13,390)
	Seeds	23/04	30/05	5/09	98	2398 (0)	5630 (2404)	17,683 (13,390)
T.subterraneum	Flowers	25/04	31/05	18/08	79	1863 (0)	4842 (2228)	16,972 (14,161)

The figures in brackets correspond to the accumulated O_3 exposure during just the 45 days the plants were exposed to the different treatments in the open-top chambers. CFA = charcoal-filtered air; NFA = Non-filtered air; NFA+ = Non-filtered air supplemented with 40 nl l^{-1}.

[a] Days after the start of the experiment.

[b] Value of the accumulated O_3 exposure index AOT40 throughout the experiment.

Two assessments were carried out to determine the seed output of *T. striatum* plants. The first evaluation was coincidental with the second flower harvest of *T. striatum* plants; at this stage the seeds were segregated from the flowers by carefully rubbing the inflorescences on a sandpaper and dried at room temperature for a month until a constant weight was reached. The second evaluation was carried out 23 days later (see Table 1), flower structures were completely dry and the seeds were collected and weighted following the aforementioned procedures.

Details on the experimental protocol, the dates the plants were introduced in the chambers, the length of O_3 exposure in the different treatments and the dates when flower and seed assessments were carried out for the different species are presented in Table 1.

2.3. *Ozone exposure indices*

As time of germination and onset of flowering differed between species (thus presenting different exposure durations), O_3 exposure indices were calculated separately for each species and harvest period. The O_3 exposure for a given treatment was calculated as an average for all mesocosm types (see Table 1). The AOT40, AOT30 (accumulated exposure over the cut-off of 30 $\mathrm{nl\,l^{-1}}$), AOT60 (accumulated exposure over the cut-off of 60 $\mathrm{nl\,l^{-1}}$) and the number of hours presenting O_3 levels above 30, 40 and 60 $\mathrm{nl\,l^{-1}}$ (NUM30, NUM40 and NUM60, respectively) were also calculated. In addition, O_3 10-h mean from 07:00 to 17:00 GMT (M10), the 24-h O_3 mean (M24), the number of days where the O_3 daily mean exceeded 33 $\mathrm{nl\,l^{-1}}$ (D33) and the number of hourly averages exceeding 100 $\mathrm{nl\,l^{-1}}$ (NUM100) were also determined.

2.4. *Statistical analyses*

Ozone and *B. maxima* competition effects on the three clover species assessed in this experiment were analysed using a two-way ANOVA for each species, considering mesocosms-type and O_3 treatment as factors. When significant differences between treatments were found ($p<0.05$), mean differences were assessed using the Tukey honest significant difference test (HSD) for unequal sample sizes. For $p<0.1$ values, differences between treatments were considered as trends. The combined analyses of the effects of these two factors on the flower biomass production of the three *Trifolium* species involved in the experiment was also performed. The validation process and the assumptions of analyses of variance followed using Shappiro-Wilk's W and Levenne tests to check the normal distribution and homogeneous variance, respectively. When non-compliance with ANOVA assumptions was found log transformations of data were carried out. The relationship between relative flower biomass and the different O_3 exposure indices was analysed using Pearson's Correlation Index and the level of significance was determined using the *t*-student test. All statistical analyses were performed using the Statistica 97 software.

3. Results and discussion

Mean air vapour pressure deficit (VPD) during the period the plants were exposed in the chambers and in the open was 0.52 and 0.57 kPa, respectively. Ozone levels in the CFA chambers was always below 15 $\mathrm{nl\,l^{-1}}$, the 10-h mean was 41 and 55 $\mathrm{nl\,l^{-1}}$ in the NFA and NFA+ chambers, respectively. The AOT40 values corresponding to the different treatments are presented in Table 1.

The three clover species involved in the experiment were very sensitive to O_3, in agreement with other experimental results reporting the great sensitivity of Mediterranean clover species (Madkour and Laurence, 2002; Bermejo et al., 2003). The experimental findings obtained for the different clover species are presented separately, a combined analysis of the response of the three clover species is also presented. Since plant response was consistently related with its early exposure to O_3 for 45 days in the OTCs, the AOT40 values presented in the text correspond to that particular period.

3.1. *T. cherleri*

Both O_3 and mesocosm-type had a significant impact ($p<0.01$ in both cases) on the flower dry weight corresponding to the first flower harvest of *T. cherleri* plants, when plant material remained in the open for 30 days after being exposed to the different O_3 treatments in the chambers. However, no significant interaction between both factors was observed (Fig 1a). Flower output in the CFA chambers was 52% greater than in the NFA and NFA+ OTCs, no differences were found between the latter two treatments. These results indicate that the exposure of this species to O_3 during its vegetative period influences its flower production, the AOT40 values related with the observed effects were 2662 and 13203 $\mathrm{nl\,l^{-1}\,h}$ for NFA and NFA+ treatment, respectively. Regarding competition, the total flower dry weight of *T. cherleri* was 48% greater when grown in monoculture than when grown together with 1 or 3 *B. maxima* plants.

Similar results were still found when the results from the second flower harvest were analysed (Fig. 1b). At this stage, the plants remained in the open for 60 days after being exposed to the different O_3 treatments in the OTCs. Ozone and mesocosm-type significantly ($p<0.001$) influenced the flower biomass of this species.

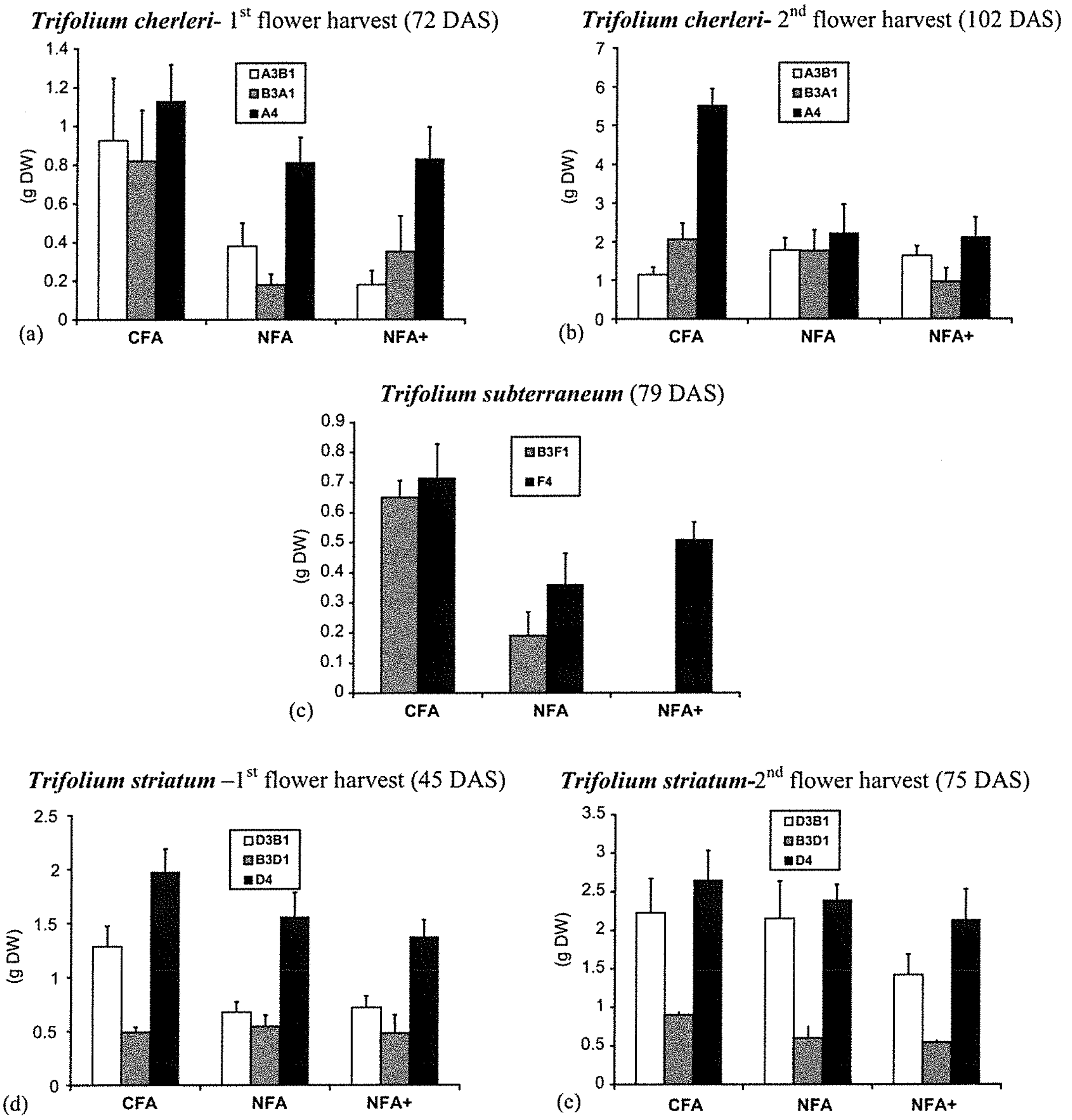

Fig. 1. Mean value and standard error for the flower production (g DW plant^{-1}) of the different mesocosms exposed to the charcoal filtered air (CFA), non-filtered air (NFA) or non-filtered air supplemented with 40 nl l^{-1} of O_3 (NFA+): (a) *T. cherleri*, 1st flower harvest (b) *T. cherleri*, 2nd flower harvest, (c) *T. subterraneum*, (d) *T. striatum*, 1st flower harvest, (e) *T. striatum*, 2nd flower harvest. B3A1, B3D1, B3F1 = 1 clover plant of *T.cherleri*, *T. striatum* or *T. subterraneum*, respectively, growing with 3 *B. maxima* plants; A3B1, D3B1 = 3 clover plants of *T. cherleri* or *T.striatum*, respectively, growing with 1 individual of *B. maxima*. A4, D4, F4 = monoculture of 4 plants of *T.cherleri*, *T.striatum* or *T.subterraneum*, respectively, DAS = Days after the start of the experiment.

Reductions of 34% and 45% were found in the flower biomass of *T. cherleri* plants grown in the NFA and NFA+ treatments, respectively. Their competition with *B. maxima* plants showed a 53% decline in their total flower biomass. A significant interaction ($p<0.001$) between O_3 exposure and mesocosm-type was found since the flower biomass of the monocultures of this species grown in the CFA treatment was 61% greater than in those mesocosms where *T. cherleri* plants were grown with one or three *B. maxima* individuals. This effect was not found in the remaining two O_3 treatments.

3.2. *T. subterraneum*

Ozone exposure induced a 60% reduction in the flower biomass of *T. subterraneum* (Fig. 1c) when NFA (AOT40 = 2228 nl l^{-1}) or NFA+ treated (14161 nl l^{-1}) plants were compared with those grown under CFA conditions ($p<0.05$). Although no O_3 exposure-mesocosm-type interactions were found for flower biomass, it tended to be greater ($p<0.1$) when grown in monoculture than when growing in competition with *B. maxima*.

3.3. *T. striatum*

This species had the shortest life-span of the three clover species tested and it was the most precocious regarding flower formation. The flower production of *T. striatum* was studied in two stages, just at the end of the 45-day period where the plant material was exposed in the OTCs to the different O_3 treatments and once the plants had remained in the open for 30 days after their exposure in the chambers.

Forty five days after the experiment was initiated (Fig. 1d) flower output was affected by both O_3 ($p<0.05$) and by its competition with *B. maxima* ($p<0.0001$); no interaction between these two factors was found. Flower biomass of the *T. striatum* plants grown in the CFA treatment was 29% greater than in those grown in the NFA (AOT40 = 2403 $\mathrm{nl\,l^{-1}\,h}$) or NFA+ chambers (AOT40 = 13389 $\mathrm{nl\,l^{-1}\,h}$). No significant differences were found between NFA and NFA+ treatments. The greatest flower production was observed when *T. striatum* was grown in monoculture, followed by those in competition with a single *B. maxima* plant (45% reduction compared with monoculture); its least flower output (69% compared with monoculture) was observed when competing with three *B. maxima* individuals.

Seventy five days after the experiment was initiated (see Fig. 1e) no significant effects were found between the different O_3 treatments. Therefore, an attenuation of O_3 effects found in the previous harvest was observed once the plants remained in the open for a month. This decline in the intensity of O_3 effects was apparently triggered by changes in the CFA plants after being exposed to ambient O_3 levels in the open, which showed very similar responses to NFA plants. The magnitude of the response of the NFA+ plants remained very similar in both harvests, although it was not significant in the second flower harvest, suggesting that the observed attenuation would be dependent on the O_3 exposure levels the plants experienced at early stages. This attenuation influenced by early O_3 exposure was also found in *T. cherleri*, where a 52% reduction in flower biomass of the NFA plants was observed in the first harvest when compared to the CFA treatment, while this difference declined to a 34% in the second flower assessment. However, this pattern was much smaller in the *T. cherleri* plants exposed in the NFA+ chambers, since the reduction in their total flower biomass when compared to the CFA treatment was 52% and 45% in the first and second harvests, respectively.

The early exposure to O_3 during 45 days in the OTCs brought about adverse effects in the seed output of the *T. striatum* plants although the two seed harvests were carried out once they remained in the open during 30 and 53 days, respectively (see Table 2). The CFA plants showed average increases for the two seed harvests of 29% and 41% when compared with NFA or NFA+ plants, respectively (see Fig. 2). The beneficial effect of charcoal filtration on the seed output was in the same range as for the total flower biomass harvested 30 days

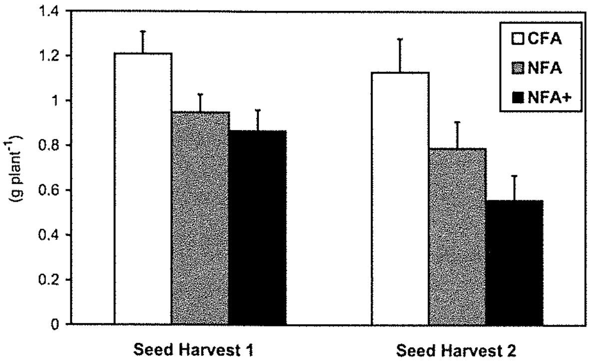

Fig. 2. Seed production of *T. striatum* plants in the two assessments that were carried out during the experiment. Each bar corresponds to the mean value and standard error of the seed output obtained for all the mesocosms under each ozone treatment (g $\mathrm{plant^{-1}}$). CFA = charcoal-filtered air; NFA = Non-filtered air; NFA+ = Non-filtered air supplemented with 40 $\mathrm{nl\,l^{-1}}$ of O_3.

Table 2

Results of the two-way ANOVA statistical test performed to assess the effects of mesocosm, ozone exposure and their interactions on the seed output of *T. striatum*

Factors	1st seed harvest (g)	2nd seed harvest (g)
Mesocosms	<0.0001	<0.0001
O_3	0.02	0.007
Mesocosms x O_3	ns	ns
B3D1		
CFA	0.44±0.05	—
NFA	0.23±0.15	—
NFA+	0.25±0.03	—
D3B1		
CFA	1.04±0.15	0.41±0.13
NFA	0.75±0.10	0.18±0.03
NFA+	0.73±0.09	0.12±0.02
D4		
CFA	1.32±0.18	1.37±0.10
NFA	1.20±0.08	0.99±0.09
NFA+	1.06±0.18	0.70±0.11

Mean ± standard error for the different mesocosms and ozone treatments are provided. CFA = charcoal-filtered air; NFA = Non-filtered air; NFA+ = Non-filtered air supplemented with 40 $\mathrm{nl\,l^{-1}}$. B3D1 = 1 clover plant of the same species growing with 3 *B. maxima* plants; D3B1 = 3 clover plants growing with 1 individual of *B. maxima*. D4 = monoculture of 4 plants of *T. striatum*.

earlier, suggesting a great relationship between these two parameters.

The *T. striatum* plants grown in competition with 3 *B. maxima* individuals showed a 69% decline in flower biomass when compared with the remaining two mesocosms. The intensity of interspecific competition induced a significant gradation in plant seed production corresponding to the first assessment, when compared with *T. striatum* monocultures: a reduction of 29% in seed weight was found when competing with a single *Briza* plant, increasing to 74% when competing with three *Briza* plants. An enhancement of this competitive effect was found at the last harvest since a 76% seed weight reduction was observed in the 3D1B mesocosm. No ozone exposure–competition interactions were found for total flower biomass or total seed weight at any stage of the experiment.

3.4. *Combined analysis of the response of the three clover species*

Since O_3 exposure determined significant reductions in the flower biomass of the three clover species a combined analysis of their response was carried out to find out whether common patterns could be derived for these species. Ozone and mesocosm significantly affected the flower production of the three species (Fig. 3). Ozone caused a significant reduction ($p<0.001$) in the flower biomass of the three species, being 27% and 34% greater in the CFA treatment than in the NFA and NFA+ treatments, respectively. Their competition with 1 and 3 *B. maxima* plants also induced 23% and 51% reductions ($P<0.0001$) in their flower biomass when compared to the values obtained in monocultures. No significant O_3 × mesocosm interactions were found.

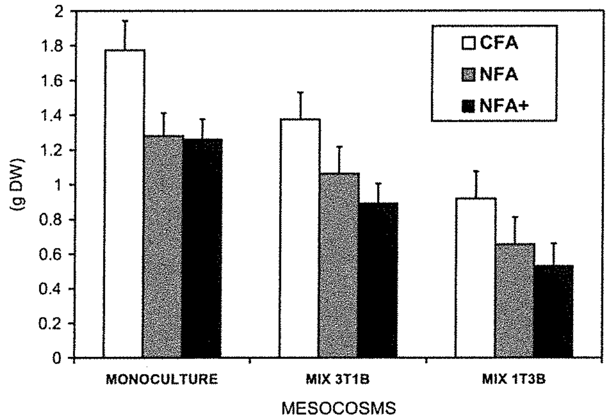

Fig. 3. Mean values and standard errors of the total flower biomass (g DW plant^{-1}) of the three clover species involved in the experiment when grown under different competitive situations and different ozone exposure regimes. Monoculture = 4 clover plants of a given species; Mix 3T1B = 3 clover plants of the same species growing with a single *B. maxima* plant; Mix 1T3B = 1 clover plant growing with 3 individuals of *B. maxima*. CFA = charcoal-filtered air; NFA = Non-filtered air; NFA+ = Non-filtered air supplemented with 40 nl l^{-1} of O_3.

The results of this experiment indicate the great sensitivity to O_3 of the three clover species assessed in this study. This is a finding of concern since it is the most predominant taxon in therophytic and siliceous pastures of the Iberian peninsula (Allué Andrade and Tella, 1986). When plants of these species were protected from ambient or above-ambient O_3 levels for 45 days, a beneficial effect on flower output was observed. The O_3-induced detrimental effects on flower biomass corresponded with AOT40 values in the range of 2200–2700 nl l^{-1} h for just 45 days, corresponding to a similar daily O_3 accumulation rate than that provided by the present O_3 critical level for the protection of seminatural vegetation of 3000 nl l^{-1} h for a three month period (Ashmore and Franzaring, 2003). However, AOT40 was not the best exposure index in explaining the response in the flower biomass found in this experiment whether considering just the period where plants remained in the OTCs or their overall exposure to this pollutant until the flowers were harvested (see Table 3). In general, those indices based on averages for different periods ranging from hours to a day (M10, M24, D33) and the indices considering 30 nl l^{-1} as a cut-off (NUM30, AOT30) performed better ($-0.86<r<-0.75$) than those indices considering cut-offs $\geqslant 60$ nl l^{-1} (AOT60 and NUM60)

Table 3
Pearson's correlation indices ($*p<0.05$, $**p<0.01$) between different ozone exposure indices and the total flower biomass of relative to control for the three clover species involved in the experiment

Ozone exposure index	OTC exposure period (45 days)	Total exposure period (45–102 days)
AOT30	−0.75**	−0.78**
AOT40	−0.66**	−0.70**
AOT60	−0.54*	−0.59*
NUM30	−0.86**	−0.83**
NUM40	−0.83**	−0.83**
NUM60	−0.61*	−0.66*
M10	−0.83**	−0.81**
M24	−0.84**	−0.81**
D33	−0.84**	−0.86**
NUM100	−0.55*	ns

AOT30, AOT40, AOT60 = accumulated exposure over the cut-off of 30, 40 and 60 nl l^{-1}, respectively, NUM30, NUM40, NUM60 = number of hours presenting O_3 levels above 30, 40 and 60 nl l^{-1}, respectively, M10 = O_3 10-hour mean from 07:00 to 17:00 GMT, M24 = 24-h O_3 mean, D33 = number of days where the O_3 daily mean exceeded 33 nl l^{-1}, NUM100 = number of hourly averages exceeding 100 nl l^{-1}.

($-0.54<r<0.61$). It appears that O_3 concentrations, regardless of their range, might be equally harmful for these species suggesting that no O_3 threshold should be considered for their protection, as previously reported by Bermejo et al. (2003) concerning the development of foliar visible injury. This fact is supported by the better performance of the index that considered the number of hours the plants were exposed to $40\,\mathrm{nl\,l^{-1}}$ (NUM40) when compared to the AOT40 index, which indirectly provides more weight to the highest O_3 concentrations (Tuovinen, 2000). This dataset should be combined with other experimental results to ascertain whether this applies to other seminatural species, too.

It can be concluded that early O_3 exposure and competition influence the reproductive ability of the studied clover species. The lack of interaction between these two factors generally found in this experiment could be explained by the great sensitivity of the clover populations used in the study and because *B. maxima* was a very strong competitor for the three clover species as derived from biomass-related parameters (data not shown). Other studies are under way involving competition with other species to evaluate whether it modulates plant response to O_3 exposure.

The results of this study have important ecological implications. Plant composition of these therophyte-prevailing systems is greatly dependent on the belowground seed pool (Peco et al., 1998a, b). Therefore the seed output of therophytes is crucial for their perpetuation. Ozone exposure appears to interfere with this process by affecting the reproductive ability of sensitive species even at rather low O_3 levels for just 45 days. Thus, O_3 exposure might affect the seed production of therophytes, which would induce an adverse impact on the species composition of these ecosystems. Field trials following O_3 exposure gradients are envisaged to test whether this experimental observation actually occurs under field conditions. Further research will concentrate in the assessment of O_3 effects on seed viability and the interactive effects of this pollutant and other environmental stresses on plant fitness.

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References

Allué Andrade, J.L., Tella, G., 1986. Los pastos españoles. II. Clasificación, composición, atributos y mejora de sus principales tipos florísticos. E.U.I.T. Forestal, Madrid, Spain.

Alonso, R., Elvira, S., Castillo, F.J., Gimeno, B.S., 2001. Interactive effects of ozone and drought stress on pigments and activities of oxidative enzymes in *Pinus halepensis*. Plant Cell and Environment 24, 905–916.

Ashmore, M., Franzaring, J., 2003. Report from the working group on semi-natural vegetation. In: Karlsson, P.E., Selldén, G., Pleijel, H. (Eds.), Establishing ozone critical levels II, UN ECE Workshop report, IVL Report B 1523, 379pp.

Ashmore, M.R., Thwaites, R.H., Aisnworth, N., Cousins, D.A., Power, S.A., Morton, J., 1995. Effects of ozone on calcareous grassland communities. Water, Air and Soil Pollution 85, 1527–1532.

Bermejo, V., Gimeno, B.S., Sanz, J., de la Torre, D., Gil, J.M., 2003. Assessment of the ozone sensitivity of 22 native plant species from mediterranean annual pastures based on visible injury. Atmospheric Environment 37, 4667–4677.

Black, V.J., Black, C.R., Roberts, J.A., Stewart, C.A., 2000. Impact of ozone on the reproductive development of plants. New Phytologist 147, 421–447.

Bungener, P., Balls, G.R., Nussbaum, S., Geissmann, M., Grub, A., Fuhrer, J., 1999. Leaf injury characteristics of grassland species exposed to ozone in relation to soil moisture condition and vapour pressure deficit. New Phytologist 142, 271–282.

Davison, A.W., Barnes, J.D., 1998. Effects of ozone on wild plants. New Phytologist 139, 135–151.

De Witt, C.T., 1960. On competition. Verslagen van Landouwkundige Onderzoekingen 66, 1–82.

EMEP, 2002. Transboundary acidification, eutrophication and ground level ozone in Europe. EMEP Report 1 and 2/2002. EMEP, Oslo, Norway.

Fuhrer, J., Shariat-Madari, H., Perler, R., Tschannen, W., Grub, A., 1994. Effects of ozone on managed pasture. II. Yield, species composition, canopy structure and forage quality. Environmental Pollution 83, 317–325.

Fuhrer, J., Ashmore, M.R., Mills, G., Hayes, F., Davidson, A.W., 2003. Ozone critical levels for semi-natural vegetation. In: Karlsson, P.E., Sellden, G., Pleijel, H. (Eds.), Establishing Ozone Critical Levels. II UNECE Workshop Report. IVL Report B 1523. IVL Swedish Environmental Research Institute, Gothenburg, Sweden, pp. 183–198.

Gimeno, B.S., Bermejo, V., Reinert, R.A., Zheng, Y., Barnes, J.D., 1999. Adverse effects of ambient ozone on watermelon yield and physiology at a rural site in eastern Spain. New Phytologist 144, 245–260.

Hamilton, N.R.S., 1994. Replacement and additive designs for plant competition studies. Review. Journal of Applied Ecology 31, 599–603.

Madkour, S.A., Laurence, J.A., 2002. Egyptian plant species as new ozone indicators. Environmental Pollution 120, 339–353.

Marañón, T., 1988. Agro-sylvo-pastoral systems in the Iberian Peninsula: Dehesas and Montados. Rangelands 10, 255–258.

Myers, N., Mittermeier, R.A., Mittermeier, C.G., da Fonseca, C.A.B., Kent, J., 2000. Biodiversity hotspots for conservation prioirities. Nature 403, 853–858.

Nussbaum, S., Geissmann, M., Fuhrer, J., 1995. Ozone exposure relationships for mixtures of perennial ryegrass and white clover depend on ozone exposure patterns. Atmospheric Environment 29, 989–995.

Palacios, M., Kirchner, F., Martilli, A., Clappier, A., Martín, F., Rodríguez, M.E., 2002. Summer ozone episodes in the greater Madrid area. Analyzing the ozone response to abatement strategies by modellling. Atmospheric Environment 36, 5323–5333.

Peco, B., Espigares, T., Levassor, C., 1998a. Trends and fluctuations in species abundance and richness in Mediterranean annual pastures. Applied Vegetation Science 1, 21–28.

Peco, B., Ortega, M., Levassor, C.M., 1998b. Similarity between seed bank and vegetation in Mediterranean grasslands: a predictive model. Journal Vegetation Science 9, 815–828.

Pineda, F.D., Nicolás, J.P., Ruiz, M., Peco, B., Bernáldez, F.G., 1981. Succesion, diversité et amplitude de niche dans les aturages du centre de la péninsule ibérique. Vegetatio 47, 267–277.

Plaza, J., Pujadas, M., Artíñano, B., 1997. Formation and transport of the Madrid ozone plume. Journal of Air and Waste Management Association 47, 666–774.

Sala, O.E., Chapin III, F.J., Armesto, J.J., Berlow, E., Bloomfield, J., Dirzo, R., Huber-Sanwald, E., Huenneke, L., Jackson, R.B., Kinzig, A., Leemans, R., Lodge, D., Mooney, H., Oesterheld, M., Poff, N.L., Sykes, M.T., Walker, B.H., Walker, M., Wall, D.H., 2000. Global diversity scenarios for the year 2100. Science 287, 1770–1774.

Snaydon, R.W., 1991. Replacement or additive design for competition studies. Journal of Applied Ecology 28, 930–946.

Tuovinen, J.P., 2000. Assessing vegetation exposure to ozone: properties of the AOT40 index and modifications by deposition modelling. Environmental Pollution 109, 361–372.

UN ECE, 1988. ECE Critical Levels Workshop. Bad Harzburg 14–18 March 1988. Final Draft Report. Unwelbundesamt, Berlin, Germany.

Warwick, T., Taylor, G., 1995. Contrasting effects of tropospheric ozone on five native herbs which coexist in calcareous grassland. Global Change Biology 1, 143–151.

3.3. MODULACIÓN DE LA RESPUESTA AL OZONO DE ESPECIES PASCÍCOLAS MEDITERRÁNEAS ANUALES POR EL INCREMENTO DE LA DISPONIBILIDAD DE NITRÓGENO

Se realizaron dos experimentos independientes con el propósito de estudiar conjuntamente el efecto del O_3 y el incremento de la disponibilidad de N simulando la entrada de depósito atmosférico en especies herbáceas anuales mediterráneas. Los experimentos se desarrollaron en una instalación de cámaras de techo descubierto (OTCs). Se consideraron tres tratamientos de O_3: aire filtrado, cuya concentración no sobrepasa las 15 ppb (CFA); aire no filtrado, que reproduce las concentraciones ambientales del contaminante (NFA); y aire no filtrado al que se le añaden 40 ppb de O_3 por encima del ambiente (NFA+). Se emplearon tres tratamientos de fertilización nitrogenada, con concentraciones máximas de 10-30-60 kg/ha. Las especies utilizadas pertenecen a dos familias de gran importancia ecológica y pascícola, tres leguminosas: *Trifolium cherleri*, *Trifoliun striatum*, y *Trifolium subterraneum;* y dos gramíneas: *Briza máxima*, y *Bromus hordeaceus*. La evaluación de efectos fue valorada utilizando parámetros de: crecimiento, calidad nutritiva, fenología y capacidad reproductiva.

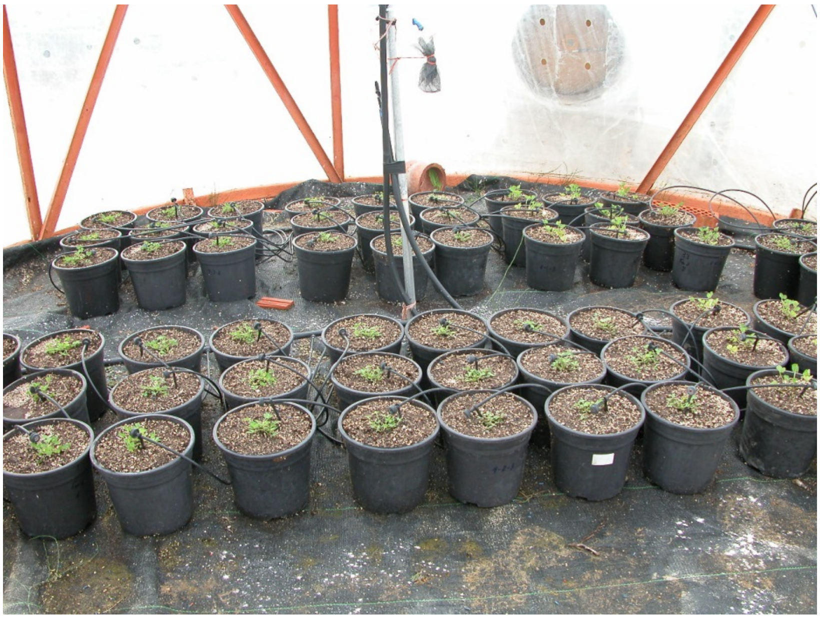

Figura 12. Especies herbáceas anuales con diferentes tratamientos de nitrógeno en el interior de una OTC (Autor: J. Sanz).

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Ozone and increased nitrogen supply effects on the yield and nutritive quality of *Trifolium subterraneum*

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Abstract

The influence of ambient ozone (O_3) concentrations and nitrogen (N) fertilization, singly and in combination, on the growth and nutritive quality of *Trifolium subterraneum* was assessed. This is an important O_3-sensitive species of great pastoral value in Mediterranean areas. Plant material was enclosed in open-top chambers (OTCs). Three O_3 levels were established: Filtered air with O_3 concentrations below 15 ppb (CFA), non-filtered air with O_3 concentrations in the range of ambient levels (NFA), and non-filtered air supplemented with 40 ppb O_3 over ambient levels (NFA+). Similarly, three N levels were defined: 5, 15 and 30 kg ha^{-1}. The increase in O_3 exposure induced a reduction of the clover aerial green biomass and an increase of senescent biomass. Ozone effects were more adverse in the root system, inducing an impairment of the aerial/subterranean biomass ratio. Compared with the CFA treatment, nutritive quality of aerial biomass was 10 and 20% lower for NFA and NFA+ treatments, respectively, due to increased concentrations of acid detergent fiber, neutral detergent fiber and lignin. The latter effect appears to be related to senescence acceleration. The increment in N supplementation enhanced the increase of ADF concentrations in those plants simultaneously exposed to ambient and above-ambient O_3 concentrations, and reduced the incremental rate of foliar senescence induced by the pollutant.

Keywords: Acid detergent fiber; Neutral detergent fiber; Crude protein; Lignin; Forage quality; Ozone; Trifolium

1. Introduction

Tropospheric ozone (O_3) is considered as one of the most phytotoxic air pollutants. The photochemical and diffusive processes involved in its generation and transport lead to the build-up of a regional air pollution problem affecting large rural areas. This issue is especially relevant in the Mediterranean area because this is the European region where the highest O_3 concentrations are attained as a result of the economical development of the region during the last decade, contributing to an increase of the emission of O_3 precursors, and the prevailing climatic conditions that enhance the photochemical processes related with the generation of this pollutant.

Ozone phytotoxic effects involve the induction of foliar injury and the alteration of plant resource acquisition and allocation patterns that might affect plant growth and yield, even when no visible injuries are

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recorded. Several studies have reported the effects of this pollutant on different organizational levels of agricultural and forestry species from the Iberian peninsula (Gimeno et al., 1999; Alonso et al., 2001). More recent studies have highlighted the great O_3 sensitivity of some therophytes from Iberian dehesa rangelands, the genus *Trifolium* being especially sensitive to this pollutant (Bermejo et al., 2003; Gimeno et al., 2004 a, b).

The emission of nitrogenous compounds from intensive agricultural and farming practices, fossil-fuel combustion or biomass burning represents the other important source of anthropogenic pollution. Although chronic deposition of N on terrestrial ecosystems may initially enhance the growth of some plants, it might overload the N assimilation capacity of those ecosystems and thus change species composition or increase plant susceptibility to other biotic and abiotic factors (Fenn et al., 2003; Fangmeier et al., 1994). The measurements of atmospheric deposition of nitrogenous compounds that have been carried out in some areas of the Iberian peninsula have reported higher deposition rates than in other European regions. For instance, Rodà et al. (2002) found N deposition rates on a broad-leaved evergreen forest located in north-eastern Spain that were in the 15–22 $kg\,ha^{-1}\,y^{-1}$ range.

Synergistic effects of air pollutants on plant performance have been reported. The high O_3 levels and N deposition rates experienced by the conifer forests in southern California induce important alterations in N and C cycles (Takemoto et al., 2001; Fenn et al., 2003). A recent study carried out at the European continental scale indicated that plants growing close to areas affected by local sources of nitrogen oxides are more prone to increase their sensitivity to O_3 (Mills et al., 2000).

Subterranean clover (*Trifolium subterraneum* L.) is a therophytic species from European Mediterranean and Atlantic coastal areas with a great forage value. It is a key species in the most valuable pastures of Iberian dehesas due to its superior forage quality and late senescence in the growing season (San Miguel, 1994). It is also the most used species for the recovery of degraded pastures, aiming to prevent soil erosion and to increase their productivity in the Iberian peninsula (Crespo and Cordero, 1998; González et al., 1998b). Its forage quality has favored its use in other regions of the world forming artificial monocultural pastures or mixed pastures with annual or perennial grasses depending on the climatic conditions (Smetham, 1999). However, *Trifolium* species have been rated as O_3 sensitive when compared with *Gramineae* species from the same ecosystems (Kochhar et al., 1980; Blum et al., 1983). In fact, an Iberian population of *T. subterraneum* has been recently classified as very sensitive to O_3 as a brief exposure to moderate levels of this pollutant induced the early development of foliar injury (Bermejo et al., 2003), an important reduction of its growth rate (Gimeno et al., 2004a) and a negative effect on its reproductive ability (Gimeno et al., 2004b). Little is known about the impact of this pollutant on its nutritive quality, an issue that has been seldom addressed for forage plants (Fuhrer et al., 1994; Muntifering et al., 2000; Powell et al., 2003). Recently, Krupa et al. (2004) have carried out a review on the O_3 effects on plant nutritive quality characteristics for ruminant animals. Exposure to elevated O_3 can directly influence nutritive quality of herbaceous vegetation as a result of altered concentrations of minerals, protein, readily available carbohydrates and cell-wall constituents (cellulose, hemicellulose and lignin). The accelerated senescence and death of plant tissue induced by O_3 can alter nutrient utilization by herbivores through reduction in voluntary intake and digestibility.

This research was performed to test whether an increased N supply can modulate the effects of O_3 exposure on the growth and nutritive quality of *T. subterraneum*.

2. Materials and methods

2.1. Plant material

T. subterraneum (L.) seeds collected in Badajoz (38°58′N, 05°50′W, 279 a.s.l.) were supplied by the Department of Pastures and Forage from the Research and Technological development Service of the *Junta de Extremadura* (Spain). Seeds were immersed for 24 h in a Germinator® solution (Agro-Orgánicos Mediterráneos S.L., Granada, Spain) to ensure homogeneous germination. When seeds were swollen they were sown in a 50% vermiculite and 50% neutral peat substrate. The seedlings were subsequently transplanted to 2.5-l pots with a 50% peat, 20% vermiculite and 30% perlite substrate, and its pH was amended by adding 4 $kg\,m^{-3}$ CaO. Each pot was supplied with 10 cm^3 of natural soil collected in a dehesa from northern Madrid area to ensure plant nodulation. Table 1 provides details on the experimental dates, from sowing to biomass harvest.

Table 1
Timetable of events during the experiment

Event	Date
Sowing	27 May
Emergence	29 May
N fertilization	29 June
Transplantation	1 July
Start of ozone exposure in OTCs	2 July
N fertilization	15 July
Harvest	31 July

2.2. Nitrogen and ozone treatments

Plant material was fertilized twice monthly at 15-day intervals with a basal fertilizer (Peters, N:P:K 4/25/35) and $(NH_4)_2NO_3$ (35%) to achieve the following N doses: 5 (N5), 15 (N15) and 30 (N30) kg ha^{-1}.

The experiment was conducted at an open-top chamber (OTC) facility located away from air pollution sources (40°41′N, 0°47′E). Three O_3 treatments were used: charcoal filtered air (CFA), non-filtered air (NFA) with near-ambient O_3 levels, and non-filtered air supplemented with 40 nl l^{-1} O_3 from 07:00 to 17:00 (GMT) 5 days per week (NFA+). Three OTC replicates were used for every O_3 treatment, and three plants per N treatment were enclosed in each chamber. Plants were irrigated with a droplet system to ensure homogeneous growth. A more detailed description of the OTC facility and its operation can be found in Alonso et al. (2001).

2.3. Assessed parameters

Aerial biomass was excised when plant material was exposed to the different O_3 treatments for 30 days. Green and senescent leaves of each plant were segregated. The green biomass was immediately weighed, and then both senescent and green leaves were dried out at 60 °C until a constant weight was reached. Similarly, roots were cautiously extracted, washed and dried at 60 °C and weighed afterwards. Root nodulation was observed in all the treatments, although no evaluation was performed regarding the number or the activity of the nodules. Also, two stems per pot were selected at random, and the number of leaves presenting foliar injury and the total number of leaves of each shoot were counted.

2.4. Chemical analyses

Composite samples were generated by pooling the dried green biomass of the three plants per treatment enclosed in each OTC. Chemical analyses were carried out in the Department of Animal Sciences of the Auburn University (USA). Dry matter (DM) concentration was determined by drying samples at 100 ºC to constant weight, and concentration of crude protein (CP = %N x 6.25) was determined by the Kjeldhal method (Association of Official Analytical Chemists, 1995). Forage concentrations of neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined sequentially according to procedures of Van Soest et al. (1991).

2.5. Statistical analyses

The effects of N fertilization and O_3 exposure on the different parameters were evaluated by performing independent two-way ANOVA analyses. When significant differences between treatments were found ($p<0.05$), mean differences were assessed using the Tukey test. ANOVA assumptions were tested using Shappiro–Wilk's W and Levenne tests to check the normal distribution and homogeneous variance, respectively. Nutritive quality-related parameters were transformed (arcosen $x^{0.5}$) to fulfill the statistical requirements. All statistical analyses were carried out using the Statistica 6.0 software.

3. Results and discussion

3.1. Climate and ozone exposure data

The average, maximum and minimum values of the different meteorological parameters and O_3 levels recorded during overall exposure period in the OTCs are presented in Table 2. The mean air temperature was 25 ºC and RH was close to 85%, with an average vapor pressure deficit (VPD) of 0.57 kPa. Mean hourly O_3 concentration in the NFA treatment was 34 nl l^{-1}, and the accumulated AOT40 value throughout the experi-

Table 2
Mean, maximum and minimum values of the meteorological parameters and ozone concentrations recorded in the open-top chambers during the 30-day exposure of plant material. Mean ± sd

	Mean	Max	Min
Temperature (°C)	24.6±2.4	32	18
RH (%)	83±14	100	30
GR (W m^{-2})	271±323	948	0
VPD (kPa)	0.57±0.53	3.04	0
O_3 CFA (nl l^{-1})	7.9±6.3	56	1
O_3 NFA (nl l^{-1})	34.4±10.8	79	10
O_3 NFA+ (nl l^{-1})	56.4±22.3	126	12

RH = air relative humidity; GR = global radiation; VPD = vapor pressure déficit; CFA = charcoal filtered air; NFA = non-filtered air; NFA+ = non-filtered air + 40 ppb O_3.

ment was 11 967 nl l^{-1} h. The mean hourly O_3 concentration in the CFA chambers was 8 nl l^{-1}, and the AOT40 for overall plant growth cycle was 39 nl l^{-1} h. The mean hourly O_3 concentration and the AOT40 value recorded for the NFA+ treatment during the same period were 56 nl l^{-1} and 11697 nl l^{-1} h, respectively. The highest O_3 hourly mean during the experimental period (126 nl l^{-1}) was recorded in the NFA+ treatment.

3.2. Visible foliar injury

Subterranean clover has been described in the literature as an O_3-sensitive species based on recorded incidence and severity of foliar injury. In fact, this species has been used as a bioindicator to assess plant responses to this pollutant across Europe within the UN/ECE ICP-Vegetation framework (Benton et al., 2000; Tonneijck and Van Dijk, 2002). In agreement with these studies, our results have indicated the O_3 sensitivity of the Spanish population used in this experiment. An accumulated exposure corresponding to an AOT40 value of 1196 nl l^{-1} h for 30 days induced the development of visible O_3 symptoms on 65% of the leaves, as an average of the different N treatments, and increased up to 81% in the NFA+ treatment. No foliar injury was recorded in the CFA plants (Table 3).

In addition to foliar injury symptoms, a 74% increase in foliar senescence was found in the NFA+ plants at the end of the exposure period compared with those grown in the other two treatments (Table 4). This type of non-specific response to O_3 has also been reported in the literature (Bergmann et al., 1999; Franzaring et al., 2000). However, in this experiment this parameter was less sensitive to O_3 exposure than O_3-specific foliar injury because no differences in leaf senescence were found between CFA and NFA plants. The senescent/green foliar biomass ratio increased over 100% in the NFA+ treatment compared with the other O_3 treatments (Table 4). This response is confirmatory of the results reported by Bermejo et al. (2003) that indicate O_3 sensitivity of this population because foliar injury was detected in the early stages of its growth, corresponding to an AOT40 value of 359 nl l^{-1} h. However, a greater extent of foliar injury was found in this experiment compared with the above-mentioned experiments. Plant responses to the pollutant depend on environmental variables affecting stomatal conductance and therefore O_3 uptake. Among them, VPD is the factor that most regulates the induction of O_3 foliar injury on subterranean clover (Balls et al., 1996) and other *Trifolium* species (Ball et al., 1998, 2000; Mills et al., 2000). In our experiment, O_3 foliar injury corresponded to AOT40 values of 95 and 1101 nl l^{-1} h when VPD was below and above 1.5 kPa, respectively. Thus, the most phytotoxic levels corresponded with those situations favoring the uptake of the pollutant (low VPD values). Nitrogen fertilization did not affect the extent of O_3 foliar injury or foliar senescence.

Table 3
Percentage of affected leaves showing O_3-induced visible injury following the exposure of plant material to the different treatments for 30 days

	Foliar injury (%)
O_3	< 0.0001
Nitrogen	ns
O_3 x nitrogen	ns
CFA N5	0
CFA N15	0
CFA N30	0
NFA N5	60.7±11.4
NFA N15	66.2±9.9
NFA N30	67.8±4.0
NFA+N5	78.1±6.9
NFA+N15	81.0±4.0
NFA+N30	84.5±5.8

The p values corresponding to the effect of each factor (O_3 and N) are presented in the first part of the table while the values (mean±se) are presented below. CFA = charcoal filtered air; NFA = non-filtered air; NFA+ = non-filtered air+40 ppb O_3; N5, N15 and N30 represent N supply at 5, 15 and 30 kg ha^{-1}, respectively.

3.3. Aerial biomass

Total aerial biomass, including both green and senescent biomass, was negatively affected by O_3 exposure and positively affected by N fertilization (Table 4). No interactive effects were detected. Reductions of 12% and 28% were found in the aerial biomass of NFA and NFA+ plants, respectively, compared with the CFA treatment. Nitrogen fertilization caused noteworthy increases in this variable; an increase of 23% was found following the 15 kg ha^{-1} application, while a 75% increase was detected in the plants fertilized with 30 kg ha^{-1} compared with the basal treatment N5. Green biomass was decreased by 15% and 56% in the NFA and NFA+ treatments, respectively, compared with CFA plants. Senescent biomass was increased by 78% in the NFA+ treatment compared with the control plants, which mitigated the negative impact of elevated O_3 on total biomass (Table 5).

Green biomass of subterranean clover was increased by 36% and 104% in the N15 and N30 treatments, respectively, compared with the N5 treatment. No significant effects of N fertilization on senescent biomass were detected.

These distinct effects of O_3 and N on green and senescent biomass of subterranean clover were reflected

Table 4
Growth-related parameters (mean ± se) corresponding to the exposure of plant material to the different O_3 treatments for 30 days

	Green biomass (g dw)	Senescence biomass (g dw)	Total aerial biomass (g dw)	Root biomass (g dw)	Total biomass (g dw)	Shoot/root ratio	Fresh/dry aerial biomass ratio
O_3	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Nitrogen	<0.0001	ns	<0.01	<0.0001	<0.0001	<0.005	<0.0001
O_3 × nitrogen	ns	ns	ns	0.1	ns	ns	ns
CFA N5	0.63 ± 0.06	0.15 ± 0.04	0.79 ± 0.06	0.47 ± 0.03	1.25 ± 0.08	1.70 ± 0.09	3.85 ± 0.25
CFA N15	0.86 ± 0.07	0.14 ± 0.02	1.00 ± 0.06	0.63 ± 0.05	1.63 ± 0.09	1.62 ± 0.10	4.37 ± 0.20
CFA N30	1.26 ± 0.09	0.14 ± 0.04	1.40 ± 0.08	0.85 ± 0.06	2.25 ± 0.12	1.69 ± 0.10	4.91 ± 0.18
NFA N5	0.53 ± 0.03	0.16 ± 0.02	0.70 ± 0.04	0.42 ± 0.03	1.11 ± 0.05	1.67 ± 0.09	3.74 ± 0.08
NFA N15	0.78 ± 0.06	0.11 ± 0.03	0.88 ± 0.06	0.47 ± 0.03	1.35 ± 0.08	1.92 ± 0.13	4.63 ± 0.16
NFA N30	1.03 ± 0.12	0.18 ± 0.03	1.21 ± 0.11	0.53 ± 0.02	1.74 ± 0.12	2.30 ± 0.19	4.67 ± 0.21
NFA + N5	0.34 ± 0.02	0.27 ± 0.01	0.61 ± 0.03	0.32 ± 0.03	0.93 ± 0.05	2.02 ± 0.15	2.65 ± 0.11
NFA + N15	0.41 ± 0.02	0.26 ± 0.01	0.67 ± 0.02	0.32 ± 0.03	0.99 ± 0.04	2.23 ± 0.16	3.06 ± 0.10
NFA + N30	0.78 ± 0.06	0.23 ± 0.01	1.02 ± 0.06	0.40 ± 0.04	1.42 ± 0.08	2.72 ± 0.26	4.01 ± 0.18

The *p* values corresponding to the effect of each factor (O_3 and N) are presented in the first part of the table while the values (mean ± se) are presented below. CFA = charcoal filtered air; NFA = non-filtered air; NFA + = non-filtered air + 40 ppb O_3; N5, N15 and N30 represent N supply at 5, 15 and 30 kg ha^{-1}, respectively.

in the senescent/green biomass ratio, which was significantly affected by both factors ($p<0.0001$). Interactive effects were detected ($p<0.05$) because N fertilization greatly compensated for the increased senescent/green biomass ratio induced by exposure to the highest O_3 levels (Fig. 1).

The fresh/dry green biomass ratio was also affected by O_3 and N fertilization although no interactions between both factors were found (Table 4). This ratio was reduced by 26% in NF+ plants compared with controls, and this effect enhanced leaf hardiness by reducing its total water content. This response has been reported when plants are exposed to water stress or following plant aging (Ribas et al., 2005). An increased nitrogen supply induced the opposite effect determining a proportional increase of the fresh/dry green biomass ratio.

3.4. *Subterranean biomass*

Subterranean biomass was reduced in the NFA and NFA+ treatments by 27% and 47%, respectively, compared with the CFA treatment; the magnitude of this effect was twice that induced by the pollutant on aerial biomass. However, the subterranean biomass of the N15 and N30 plants was increased by 17% and 48%, respectively, compared with the N5 plants. The magnitude of this effect was half of that induced by N fertilization on aerial biomass. These findings indicate that both factors not only elicited opposite responses on plant performance, but they also affected different targets because N fertilization most affected plant aerial biomass while the greatest effect of O_3 exposure was on subterranean biomass. Although non-significant $O_3 \times N$ interactions were detected ($p = 0.1$), increased N supply tended to intensify the O_3 effect on subterranean biomass because increments were recorded in the CFA treatment with increasing N fertilization doses, whereas no variations in subterranean biomass of the NFA and NFA+ treatments were observed in spite of increased N fertilization. These contrasting effects of both factors were translated into significant alterations of the shoot/root ratio. Increments of 17% and 39% for this ratio were observed in the NFA and NFA+ treatments, respectively, compared with control plants. Significant effects of N fertilization on this parameter were only found at the highest dose (N30), with an increment of 24% compared with the N5 treatment.

The high sensitivity of the subterranean biomass of this species to O_3 exposure agrees with the results from a screening study carried out by Gimeno et al. (2004a), who reported that *T. subterraneum* exhibited the largest increase in its shoot/root ratio (38%) among a total of 22 therophytes from dehesa grasslands. Several studies have also reported similar alterations in shoot/root ratio following plant exposure to this pollutant (Reiling and

Table 5
Ozone (O_3) and nitrogen supply (N) effects on nutritive quality-related parameters of *Trifolium subterraneum* at the end of the exposure period (30 days)

	CP (%)	NDF (%)	ADF (%)	Lignin (%)
O_3	< 0.001	< 0.001	< 0.001	< 0.001
Nitrogen	< 0.001	ns	ns	ns
O_3 x nitrogen	ns	ns	< 0.05	ns
CFA N5	9.42±0.69	29.19±0.53	17.78±0.28a	0.35±0.06
CFA N15	10.86±0.43	29.71±0.62	17.67±0.27a	0.44±0.07
CFA N30	14.20±0.70	29.99±0.41	17.09±0.20a	0.38±0.14
NFA N5	9.87±0.50	31.12±0.49	18.97±0.22ab	0.57±0.07
NFA N15	12.70±0.49	31.5±0.54	18.68±0.21ab	1.11±0.10
NFA30 4	16.31±0.84	34.51±1.30	19.79±0.50bc	1.82±0.38
NFA+N5	12.47±0.38	35.62±0.06	21.06±0.10c	1.72±0.32
NFA+N15	14.26±0.63	36.31±1.17	20.59±0.47c	1.91±0.21
NFA+N30	17.48±1.02	35.87±0.72	19.71±0.13bc	1.80±0.22

The upper part of this table shows the results of the two-way ANOVA test that was carried out to evaluate the effects of ozone and nitrogen, singly and in combination, on the assessed parameters. The lower part indicates the mean values and their standard deviations corresponding to the different treatments. CP = crude protein; NFD = neutral detergent fiber; FAD = acid detergent fiber; CFA = charcoal filtered air; NFA = non-filtered air; NFA+ = non-filtered air+40 ppb O_3; N5 = total N supply of 5 kg ha^{-1} y^{-1};N15 = total N supply of 15 kg ha^{-1} y^{-1}; N30 = total N supply of 30 kg ha^{-1} y^{-1}. Different letters indicate significant effects for O_3 and N interactions ($p<0.05$).

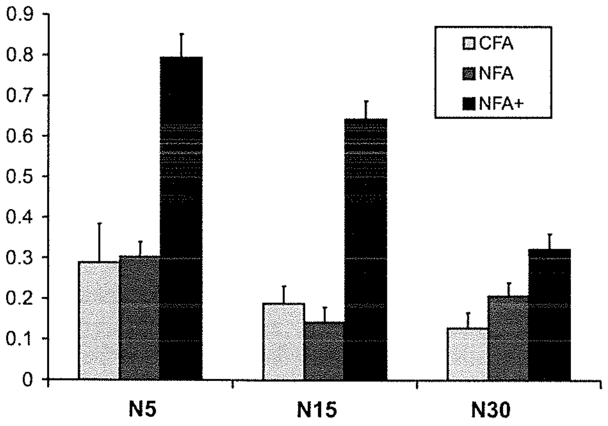

Fig. 1. Senescent/green aerial biomass ratio found in the different ozone and nitrogen supply treatments. Mean and standard errors are presented. CFA = charcoal filtered air; NFA = non-filtered air; NFA+ = non-filtered air+40 ppb O_3; N5 = total N supply of 5 kg ha^{-1} y^{-1}; N15 = total N supply of 15 kg ha^{-1} y^{-1}; N30 = total N supply of 30 kg ha^{-1} y^{-1}.

Davison, 1992; Franzaring et al., 2000). Blum et al. (1981) observed that O_3 reduced root biomass and total non-structural carbohydrates of roots, suggesting that O_3 would reduce the survival of clover during periods of stress because roots serve as the primary source of energy reserves. This fact might be of ecological relevance for Mediterranean therophytes because seed production usually coincides with severe drought during summer and, as a result, a significant amount of photosynthate in these species usually accumulates in the root system to overcome stressful periods when soil moisture and nutrient availability are limited. This structural change may affect the competitive ability of these species (Aers et al., 1991; Franzaring et al., 2000).

3.5. Nutritive quality

Ozone exposure and N fertilization affected all the assessed nutritive parameters of *T. subterraneum*, although only acid detergent fiber concentration (ADF) was modulated by the combination of both factors.

Crude protein concentration (CP) in green biomass of subterranean clover was increased by both O_3 exposure and N fertilization. A significant increase in CP concentration of 28% was found in the NFA+ treatment compared to the controls, which might be explained by the reduction of green biomass induced by O_3 that would cause a concentration effect on this parameter. This effect has also been found by the authors in other clover species (data not presented), and agrees with the findings of Flagler and Youngner (1985) who reported an increase in CP concentration at the expense of yield in tall fescue.

Increases of 19% and 51% in concentration of CP were found in the N15 and N30 treatments, respectively, compared with the N5 treatment, which could be related to enhancement of CO_2 assimilation and therefore with plant N concentrations.

Nitrogen fertilization did not have any effect on concentrations of ADF, neutral detergent fiber (NDF) or lignin, in agreement with González (1998a). However, ADF and NDF concentrations were 9% and 19% higher, respectively, in both NFA and NFA+ plants compared with controls. Lignin concentrations were greatly increased by O_3 exposure, with increments of 200% and 366% in the NFA and NFA+ plants compared with control plants. Thus, increases in lignin concentration were relatively greater than those of other plant cell-wall constituents, consistent with generalized plant response to environmental stress. These results are in contrast with findings reported by Fuhrer et al. (1994) regarding the response of CP and crude fiber concentrations in a mixed fescue–clover pasture. However, Powell et al. (2003) and Muntifering et al. (2000) observed increased concentrations of ADF, NDF and lignin in *Lespedezea cuneata* and *Paspalum notatum*, following exposure to O_3; nevertheless, their reported increments in lignin concentrations were less pronounced than those found in our experiment.

The NDF fraction of forage consists of partially and variably digestible cell-wall constituents (primarily cellulose, hemicellulose, lignin, silica and insoluble N) and is inversely related to its voluntary intake in vivo, whereas ADF consists of the least digestible cell-wall constituents (NDF less hemicellulose) and is inversely related to digestibility (Van Soest, 1994). Thus, the lower the NDF and ADF values of forage, the greater its digestible DM intake. Thus, practically speaking, low NDF and ADF values are desirable in forage from the standpoint of its nutritive quality for ruminant herbivores. Relative feed value (RFV) integrates DM intake and digestibility predicted from concentrations of NDF and ADF, respectively, into a single index of nutritive quality that can be used to comparatively evaluate different forages (Rohweder et al., 1978). Compared with the CFA treatment, RFV of aerial biomass was 10% and 20% lower for NFA and NFA+ treatments, respectively. Structural changes occur in the cell walls of *Trifolium* species through their phenological stages, causing alterations in their ADF, NDF and lignin concentrations and reducing their nutritive quality with plant maturity (Ayres et al., 1998; Mulholland et al., 1996). The O_3-induced increases in ADF, NDF and lignin concentrations observed in our experiment could be associated with acceleration of senescence processes triggered by this pollutant that would also be reflected in decreased green/dry biomass ratio. In fact, induction of early senescence has also been found in other Mediterranean therophytes, with reductions in foliar pigment content (Madkour and Laurence, 2002).

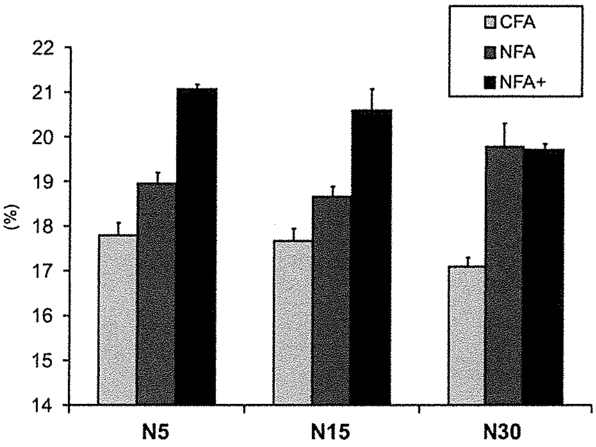

Fig. 2. ADF concentration (%) in the different ozone and nitrogen supply treatments. Mean and standard errors are presented. CFA = charcoal filtered air; NFA = non-filtered air; NFA+ = non-filtered air + 40 ppb O_3; N5 = total N supply of 5 kg ha^{-1} y^{-1}; N15 = total N supply of 15 kg ha^{-1} y^{-1}; N30 = total N supply of 30 kg ha^{-1} y^{-1}.

N fertilization significantly enhanced the detrimental effects of O_3 exposure on ADF concentrations ($p < 0.05$) (Fig. 2). Increased ADF concentrations of the N5 and N15 plants were only observed when plants were exposed to above-ambient O_3 levels. However, when plants were fertilized with a higher N dose (N30), significant increases in this parameter were observed when plants were exposed to ambient O_3 levels.

The overall results found in our experiment indicate the great O_3 sensitivity of *T. subterraneum*, because brief exposure to this pollutant corresponding to AOT40 levels of ca. 1.196 ppb h is sufficient to induce deleterious effects in its growth and nutritive quality. Complex interactive effects of both O_3 and N were observed. Nitrogen fertilization modulated plant response to the pollutant by increasing plant green biomass and reducing senescence of its aerial biomass; however, it enhanced detrimental effects of O_3 on nutritive quality and on photosynthate allocation to roots. The latter effect may have ecological consequences because root development is directly related to seed output of Mediterranean therophytes and therefore with their survival and competitive potential (Peco et al., 1998). The effect on forage quality is sufficient to have nutritional and economic implications to utilization of *T. subterraneum* by ruminant herbivores. New experimental studies involving other Mediterranean therophytes are being carried out to test whether the observed interactive effects of O_3 exposure and N fertilization on *T. subterraneum* could be generalized.

4. Conclusions

This study has indicated the great O_3 sensitivity of *T. subterraneum*. A 30-day exposure to the pollutant induced reductions in its aerial and subterranean biomass, and altered its shoot/root ratio through a more pronounced effect on root biomass. Significant detrimental effects on its nutritive quality were also found that could be associated with accelerated senescence-related processes. Nitrogen fertilization intensified O_3 effects on concentration of the lignocellulose fraction of the plant cell wall (ADF), while it partially ameliorated the detrimental effects of the pollutant on green/senescent biomass ratio.

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References

Alonso, R., Elvira, S., Castillo, F.J., Gimeno, B.S., 2001. Interactive effects of ozone and drought stress on pigments and activities of oxidative enzymes in *Pinus halepensis*. Plant Cell and Environment 24, 905–916.

Aers, R., Boot, R.G.A., van der Aart, P.J.M., 1991. The relation between above- and below-ground biomass allocation patterns and competitive ability. Oecologia 87, 551–559.

Association of Official Analytical Chemists, 1995. Official Methods of Analysis (16th ed.). Association of Official Analytical Chemists, Washington, DC.

Ayres, J.F., Nandra+, K.S., Turner, A.D., 1998. A study of the nutritive value of white clover (*Trifolium repens* L.) in relation to different stages of phenological maturity in the primary growth phase in spring. Grass and Forage Science 53, 250–259.

Ball, G., Benton, D., Palmer-Brown, J., Fuhrer, J., Skärby, L.S., Gimeno, B.S., Mills, G., 1998. Identifying factors which modify the effects of ambient ozone on white clover (*Trifolium repens* L.) in Europe. Environmental Pollution 103, 7–16.

Ball, G., Benton, J., Palmer-Brown, D., Fuhrer, J., Skärby, L., Gimeno, B.S., Mills, G., 2000. Identification of non-linear influences on the seasonal ozone dose response of sensitive and resistant clover biotypes using artificial neural networks. Ecological Modelling 129, 153–168.

Balls, G.R., Palmer-Brown, D., Sanders, G.E., 1996. Investigating microclimatic influences on ozone injury in clover (Trifolium subterraneum) using artificial neural networks. New Phytologist 132, 271–280.

Benton, J., Fuhrer, J., Gimeno, B.S., Skärby, L., Palmer-Brown, D., Ball, G., Roadknight, C., Mills, G., 2000. An international cooperative programme inidicates the widespread occurrence of ozone injury on crops. Agriculture. Ecosystems and Environment 78, 19–30.

Bergmann, E., Bender, J., Weigel, H.-J., 1999. Ozone threshold doses and exposure-response relationships for the development of ozone injury symptoms in wild plant species. New Phytologist 144, 423–435.

Bermejo, V., Gimeno, B.S., Sanz, J., de la Torre, D., Gil, J.M., 2003. Assessment of the ozone sensitivity of 22 native plant species from Mediterranean annual pastures based on visible injury. Atmospheric Environment 37, 4667–4677.

Blum, U., Smith, G.R., Fites, R.C., 1981. Effects of multiple O_3 exposures on carbohydrate and mineral contents of ladino clover. Environmental and Experimental Botany 22, 143–154.

Blum, U., Heagle, A.S., Bums, J.C., Linthurst, R.A., 1983. The effects of ozone on fescue–clover forage: Regrowth, yield and quality. Environmental and Experimental Botany 23, 121–132.

Crespo, M.C., Cordero, S.A., 1998. Productividad y persistencia de ecotipos autóctonos de trébol subterráneo de la dehesa salmantina en condiciones de pastoreo. Pastos XXVIII 1, 89–95.

Fangmeier, A., Hadwiger-Fangmeier, A., Van der Eerden, L., Jäger, H.J., 1994. Effects of atmospheric ammonia on vegetation–A review. Environmental Pollution 86, 43–82.

Fenn, M.E., Poth, M., Bytnerowicz, A., Sickman, J.O., Takemoto, B.K., 2003. Effects of ozone, nitrogen deposition and other stressors on montane ecosystmes in the Sierra Nevada. In: Ozone Air Pollution in the Sierra Nevada: Distribution and Effects on Forests. Bytnerowicz, A., Arbaugh.

Flagler, R.B., Youngner, V.B., 1985. Ozone and sulfur dioxide effects on tall fescue: II Alteration of quality constituents. Journal of Environmental Quality 14, 463–466.

Franzaring, J., Tonneijck, A.E.G., Koijman, A.W.N., Dueck, Th.A., 2000. Growth responses to ozone in plant species from wetlands. Environmental and Experimental Botany 44, 39–48.

Fuhrer, J., Shariat-Madari, R., Perler, R., Tschannen, W., Grub, A., 1994. Effects of ozone on managed pasture: II Yield, species composition, canopy structure and forage quality. Environmental Pollution 86, 307–314.

Gimeno, B.S., Bermejo, V., Reinert, R.A., Zheng, Y., Barnes, J.D., 1999. Adverse effects of ambient ozone on watermelon yield and physiology at a rural site in Eastern Spain. New Phytologist 144, 245–260.

Gimeno, B.S., Bermejo, V., Sanz, J., de la Torre, D., Elvira, S., 2004a. Growth response to ozone of annual species from Mediterranean pastures. Environmental Pollution 132, 297–306.

Gimeno, B.S., Bermejo, V., Sanz, J., de la Torre, D., Gil, J.M., 2004b. Assessment of the effects of ozone exposure and plant competition on the reproductive ability of three therophytic clover species from Iberian pastures. Atmospheric Environment 38, 2295–2303.

González, A., 1998a. Manejo de la pradera de raigrás inglés y trébol blanco en primavera y uso de nitrógeno para el primer corte. En: *Actas de la XXXVIII Reunión Científica de la SEEP*, 101-104. Soria (España).

González, F., Paredes, J., Prieto, P. M., Murillo, M., 1998b. Recuperación de los pastos degradados en la dehesa extremeña. Técnicas de revegetación con prevención de la erosión. Actas de la XXXVIII Reunión Científica de la SEEP.

Kochhar, M., Blum, U., Reinert, R.A., 1980. Effects of O_3 and (or) fescue on ladino clover: Interactions. Canadian Journal of Botany 58, 241–249.

Krupa, S., Muntifering, R., Chappelka, A., 2004. Effects of ozone on plant nutritive quality characteristics for ruminant animals. The Botanica 54, 1–12.

Madkour, S.A., Laurence, J.A., 2002. Egyptian plant species as new ozone indicators. Environmental Pollution 120, 339–353.

Mills, G., Ball, G., Hayes, F., Fuhrer, J., Skärby, L., Gimeno, B.S., De Temmerman, L., Heagle, A.S., 2000. Development of a multi-factor model for predicting the critical level of ozone for biomass reduction for white clover. Environmental Pollution 109, 533–542.

Mulholland, J.G., Nandra, K.S., Scott, G.B., Jones, A.W., Coombes, N.E., 1996. Nutritive value of subterraneum clover in a temperate enviroment. Australian Journal of Experimental Agriculture 36, 803–814.

Muntifering, R.B., Crosby, D.D., Powel, M.C., Chappelka, A., 2000. Yield and quality characteristics of bahiagrass (*Paspalum notatum*) exposed to ground-level ozone. Animal Feed Science and Technology 84, 243–256.

Peco, B., Espigares, T., Levassor, C., 1998. Trends and fluctuations in species abundance and richness in Mediterranean annual pastures. Applied Vegetation Science 1, 21–28.

Powell, M.C., Muntifering, R.B., Lin, J.C., Chappelka, A.H., 2003. Yield nutritive quality of seriacea lespedeza (*Lespedeza cuneata*) and little bluestem (*Schizachyrium scoparium*) exposed to ground-level ozone. Environmental Pollution 122, 313–322.

Reiling, K., Davison, A.W., 1992. Effects of a short ozone exposure given at different stages in the development of *Plantago major* L. New Phytologist 121, 643–647.

Ribas, A., Peñuelas, J., Elvira, S., Gimeno, B.S., 2005. Ozone exposure induces the activation of leaf senescence-related processes and morphological and groth changes in seedlings of Mediterranean tree species. Atmospheric Environment 134, 291–300.

Rodà, F., Avila, A., Rodrigo, A., 2002. Nitrogen deposition in Mediterranean forests. Environmental Pollution 118, 205–213.

Rohweder, D.A., Barnes, R.F., Jorgensen, N., 1978. Proposed hay grading standards based on laboratory analyses for evaluating quality. Journal of Animal Science 47, 747–759.

San Miguel, A., 1994. La dehesa española. Origen, topología, características y gestión. E.T.S.I.M., Madrid, Spain., 96pp.

Smetham, M.L., 1999. The ecology and use of subterraneum clover (*Trifolium subterraneum* L.) as a pasture legume. An Australasian perspective. Pastos. XXIX (1), 5–63.

Takemoto, B.K., Bytnerowicz, A., Fenn, M.E., 2001. Current and future effects of ozone and atmospheric nitrogen deposition on California's mixed conifer forest. For.Ecol.-Manag. 144, 159–173.

Tonneijck, A.E.G., Van Dijk, C.J., 2002. Injury and growth response of subterranean clover to ambient ozone as assessed by using ethylenediurea (EDU). Three years of plant monitoring at four sites in The Netherlands. Environmental and Experimental Botany 48, 33–41.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74, 3583–3597.

Van Soest, P.J., 1994. Nutritional Ecology of the Ruminant, 2nd Ed. Comstock, Ithaca, NY.

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Ozone sensitivity of the Mediterranean terophyte *Trifolium striatum* is modulated by soil nitrogen content

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Abstract

The influence of nitrogen (N) fertilization on the sensitivity to ozone (O_3) has been studied using the O_3 sensitive species *Trifolium striatum*, an annual species frequently found in therophitic grasslands of the dehesas in the central Iberian Peninsula. The experiment was carried out in open-top chambers using three different O_3 exposure treatments: charcoal-filtered air (CFA), non-filtered air (NFA) and non-filtered air plus $40\,\mathrm{nl\,l^{-1}}$ added over ambient concentrations (NFA+). After 30 days of exposure to the different treatments, plants were placed under ambient conditions until seed ripeness was completed. Three N fertilization treatments were established receiving final doses of 10 (low), 30 (medium) or 60 (high) $\mathrm{kg\,N\,ha^{-1}}$ throughout the growing period divided into four proportional doses at every 15 days. Ozone affected phenology and reduced total aerial and flower biomass and seed production in NFA and NFA+ treatments. Carry-over effects of O_3 on the reproductive ability of this species were found resulting in reductions in flower biomass and seed production detected 1 month after O_3 exposure. By contrast, N fertilization increased aerial and flower biomass. Significant $O_3 \times N$ interactive effects were detected since N fertilization counterbalanced O_3-induced effects only when plants were exposed to moderate O_3 levels (NFA) but not under high O_3 concentrations (NFA+). The observed O_3 effects on reproductive ability of *T. striatum* might determine changes in species viability and future diversity of the ecosystem.

Keywords: Grassland; Dehesa; Mediterranean pastures; Ozone critical levels; Clover; Biomass production; Seed production

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1. Introduction

Ozone (O_3) is one of the most relevant air pollutants in the Mediterranean region, where high concentrations are recorded over vast areas, mainly in rural and suburban zones. In the last two decades, evidences of O_3-induced phytotoxic effects on horticultural and forest tree species have been accumulated in this region (Fumagalli et al., 2001; Fuhrer and Booker, 2003). Recent research has been conducted to assess O_3 effects on semi-natural Mediterranean vegetation, mainly focused on therophytic dehesa grasslands of central Iberian Peninsula. Plant species richness of these ecosystems is quite remarkable and the dehesas represent an example of sustainable management of natural resources including agricultural, timber and extensive livestock exploitation (Peco et al., 1998a). *Trifolium*, the predominant taxon in dehesa grasslands (Allué Andrade and Tella, 1986), is considered

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as very sensitive to O_3 (Nebel and Fuhrer, 1994). *Trifolium striatum* was the most O_3 sensitive species out of 22 therophytes from dehesa grasslands based on visible injury (Bermejo et al., 2003), adverse effects on relative-growth rate (Gimeno et al., 2004a) or reproductive ability (Gimeno et al., 2004b). Moreover, O_3 caused detrimental effects on the nutritive quality of some species of the *Trifolium* genus (Sanz et al., 2005), an effect that could be of great interest due to its livestock feeding value (Krupa et al., 2004).

Besides direct O_3-induced effects, the so-called carry-over effects have been described in plants once O_3 exposure has ceased. These types of effects have been widely described in woody species (Elvira et al., 1998). Recently, Gimeno et al. (2004b) observed O_3-induced decreases of total floral production in three annual clover species 1 month after the cessation of O_3 exposure.

Ozone-induced effects on vegetation might be modified by elevated nitrogen (N) inputs in natural ecosystems. Traditional use of dehesas as extensive livestock exploitation produces important but scattered N inputs allowing a mosaic of small areas with different species and soil composition. In the last years emissions of nitrogenous compounds resulting from intensive agricultural and farming practices, fossil-fuel combustion or biomass burning, continue increasing, adding extra N to the ecosystems. Although chronic N deposition usually enhances growth, it might overload the N assimilation capacity of the ecosystem causing changes in plant community composition, disruption in nutrient cycling or increasing plant susceptibility to other biotic or abiotic factors (Fenn et al., 2003). Nitrogen enrichment is considered as one of the main driving forces in altering Mediterranean terrestrial ecosystems (Sala et al., 2000).

The combined effects of N enrichment and O_3 exposure have caused major physiological disruption and significant alterations in the carbon and N cycles of Californian forests (Takemoto et al., 2001; Fenn et al., 2003). Similarly, the work carried out by Mills et al. (2000) in the framework of a pan-European study indicated that O_3 phytotoxicity in *Trifolium repens* was enhanced at those sites close to local sources of nitrogen oxides (NO_x). Sanz et al. (2005) described the complexity of the interactive effects of O_3 and N in *Trifolium subterraneum*, pointing out that N supplementation enhanced detrimental effects of O_3 on nutritive quality but ameliorated O_3 effects on green biomass production.

The co-occurrence of high atmospheric N inputs and high O_3 concentrations in the dehesa ecosystems located in the Northern Madrid region has been reported by several authors (Plaza et al., 1997; Pujadas et al., 2000; Palacios et al., 2002). Therefore, an experiment was carried out to test the hypotheses that an early exposure to O_3 can induce carry-over effects on yield and reproductive ability of the O_3 sensitive species *T. striatum* and that N enrichment might enhance O_3-induced phytotoxic effects, potentially affecting the persistence of this species in the community.

2. Materials and methods

2.1. Plant material

The germ plasm bank of the Agriculture and Environment Department of the Extremadura Autonomous Community (Spain) supplied *T. striatum* seeds collected at the central-western area of the Iberian Peninsula (38°55′N, 05°06′W). Seeds were immersed during 24 h in a Germinator® solution (Agro-Orgánicos Mediterráneos S.L., Granada, Spain) to ensure homogeneous germination. When seeds were swollen they were sown in a 50% neutral peat and 50% vermiculite substrate. Seedlings were transplanted to 2.5 l pots with a 50% peat, 30% vermiculite and 20% perlite substrate. A low N fertilizer (Peters, NPK: 4/25/35 plus microelements) was provided to prevent plant material from suffering nutritional deficiencies. Two sets of 90 plants were prepared to allow two harvests. Plants were irrigated with a drip irrigation system to ensure adequate and homogeneous water availability. The timetable of the experimental protocol is presented in Table 1.

2.2. Nitrogen and O_3 treatments

Four additional applications of different doses of NH_4NO_3 were performed every 15 days aiming to reach N integrated doses of 10, 30 or 60 $kg\,ha^{-1}$, corresponding to the low, medium and high N treatments, respectively (Table 1). These N integrated doses were selected to reflect the natural variability found in N inputs in the dehesa soils of the Iberian Peninsula, considering both N entered via manure (normal stocking rate is $<1\,cow\,ha^{-1}$) and expected atmospheric N deposition (15–22 $kg\,N$ deposited $ha^{-1}\,yr^{-1}$ in broadleaved evergreen forests in NE Spain reported by Rodà

Table 1
Timetable of events during the experiment

Event	Date	DSE
Sowing	27 May	–
N fertilization	29 June	–
Transplantation	1 July	–
Start of ozone exposure in OTCs	2 July	1
N fertilization	15 July	14
N fertilization	29 July	28
First harvest	31 July	30
End of NFA+ treatment	8 August	39
End of OTCs exposure—start of ambient exposure	12 August	42
N fertilization	21 August	51
Final harvest NFA+	26 August	56
Final harvest NFA	2 September	63
Final harvest CFA	6 September	67

DSE, days from the start of experiment; NFA+, non-filtered air supplemented with 40 nl l^{-1} O_3; NFA, non-filtered air; CFA, charcoal filtered air.

et al., 2002). These values also agree with the available N estimated in soils of dehesas in central Spain based on organic matter content (Montoya et al., 1988; The Fertilizer Institute, 1982). Plant nodulation was achieved by supplying 10 cm^3 pot^{-1} of natural soil from a dehesa close to Madrid.

Starting the day after transplantation, seedlings were exposed for 30 days to the different O_3 treatments in an open-top chamber (OTC) facility far from any important source of air pollution (40°41′N, 0°47′E) described in Alonso et al. (2001). *T. striatum* seedlings were exposed to three O_3 treatments: charcoal-filtered air (CFA), non-filtered air (NFA) and non-filtered air supplemented with 40 nl l^{-1} O_3 from 07:00 to 17:00 h (GMT) 5 day-week^{-1} (NFA+). Ozone concentrations in the NFA+ treatment were in the range of those experienced by this species under field conditions. An automatic system provided continuous monitoring of O_3, sulfur dioxide and NO_x concentrations in the different treatments. The AOT40, AOT30 and AOT60 cumulative O_3 exposure indexes (accumulated exposure over the cut-off of 30, 40 and 60 nl l^{-1}, respectively) and 24-h O_3 mean were calculated for each harvesting period. The official network of the Catalan Meteorological Service (Spain) provided data of wind speed and direction, air temperature and relative humidity (RH), and global radiation. Three plants for each N treatment and harvest were introduced into each chamber and three replicated OTCs were used per O_3 treatment, thus using nine plants per each combination of O_3 exposure and N treatment per harvest.

After 30 days of O_3 exposure and two N applications, half of the plants were harvested (early harvest). Since N application was carried out every 15 days from the start of the experiment, only two N applications were performed prior to early harvest, corresponding to actual N doses of 5, 15 and 30 kg ha^{-1}. The remaining plants were exposed to the open while receiving two more N applications completing the doses of 10, 30 or 60 kg ha^{-1} for low, medium and high N treatments, respectively.

2.3. Growth and reproductive ability

Aerial and root biomass of plant material were determined at two times. The first harvest was performed after 30 days of exposure to the different O_3 treatments inside the chambers (early harvest). The late harvest was carried out when 90% of the plants of a given O_3 treatment finished their life cycle and presented fully ripened seeds (Table 1). This stage was achieved when all plant material remained in the open for about 30 days. This procedure enabled to assess treatment effects on flower biomass and seed weight when plant material reached the same phenological stage in all the O_3 treatments.

The aerial biomass production was obtained by excising the plants at the surface of the substrate and drying it at 60 °C until constant weight. Green and senescent leaves were separated only in the first harvest and the green/senescent biomass ratio was determined. Roots were carefully extracted, washed and dried at 60 °C and weighed afterwards. Aerial/subterranean dry biomass ratio was calculated to evaluate potential shifts in photosynthate distribution.

The reproductive ability of the plant was evaluated at two stages: at the early harvest, counting the number of developed flowers per plant, and at the late harvest, when seed ripening had occurred, weighing flower and seed production. Root nodulation was observed in all the treatments, although no evaluation was performed regarding the number or the activity of the nodules.

2.4. Statistical analyses

Ozone and N effects were tested by performing two-way ANOVA analyses for each parameter assessed. When significant differences between treatments were detected ($p<0.05$), mean differences were assessed using the Tukey honest significant difference test. Normal probability plots and scatter plots of residuals were used to test data normality. Levenne test was applied to check variance homocedasticity. When non-compliance with ANOVA assumptions was observed, data were log or square-root transformed. All statistical analyses were carried out using Statistica 5.1 software (StatSoft, Inc., USA).

3. Results and discussion

3.1. Meteorological and ozone exposure data

Air RH remained high during all the experiment, being over 80% during 16 h day^{-1} and decreasing during midday to values always over 60%. Mean daily values of RH were 83% and 84% for early and late harvests, respectively. Air temperature was in the range of 20–30 °C during the whole assay with mean values of 24.6 °C for the first harvest period and 23.8 °C for the last period. Although mean RH and temperature for both periods were similar, atmospheric conditions were slightly more stressful during the first harvest, when plants were exposed to O_3 treatments inside the chambers, with vapor pressure deficit (VPD) values reaching 1.01 kPa at midday while the highest VPD was 0.8 kPa during the second period.

Ozone was the most abundant air pollutant in this area. Sulfur dioxide and NO_x were within the detection level ranges of the monitors used. Ozone levels in the NFA+ treatment were in the range of values recorded in the most polluted areas of the region (Millán et al., 2000). The O_3 exposure indexes calculated for the different harvests and treatments are presented in Table 2. AOT indexes for the whole experimental period must be calculated adding early plus late harvest values. Current critical level for semi-natural communities dominated by annual species is 3000 nl l^{-1} h expressed as the AOT40 calculated over a 3 months period or growing season if shorter (UNECE, 2004). In this experiment, the critical level was not exceeded in either CFA or NFA treatments but the accumulated exposure in NFA+ exceeded by three times the critical level during the first month of O_3 exposure inside the chambers and by 4 times by the end of the experiment.

3.2. Aerial biomass

Growth related parameters per O_3 and N treatments together with results of the ANOVA analysis, considering the early and late harvest, are shown in Tables 3 and 4, respectively.

3.2.1. Early harvest

After 30 days of exposure to the different O_3 treatments inside the OTCs, O_3-induced reductions in the total aerial biomass of *T. striatum* in NFA and NFA+ treatments by 19% and 33%, respectively, when compared to controls. Even higher reductions were found considering only the green biomass, 26% and 44% for NFA and NFA+ treatments, respectively (average values of all N treatments; Table 3). On the other hand, O_3 increased the rate of foliar senescence by 25% and 44% in NFA and NFA+ treatments, respectively, when compared to CFA plants. The increase in senescent biomass is a non-specific response that can develop due to multiple stresses, but it has been frequently described in plants exposed to O_3 enriched atmospheres (Franzaring et al., 2000; Sanz

Table 2
Ozone exposure indexes per harvest

	Period (dd/mm–dd/mm)	No. of days	24 h O_3 mean (nl l^{-1})	AOT30 (nl l^{-1} h)	AOT40 (nl l^{-1} h)	AOT60 (nl l^{-1} h)
Early harvest						
CFA	01/07–31/07	31	8	115	39	0
NFA	01/07–31/07	31	34	3375	1196	157
NFA+	01/07–31/07	31	56	13,818	10,190	4053
Late harvest						
CFA	01/08–06/09	37	22	2743	917	25
NFA	01/08–02/09	33	29	2247	599	1
NFA+	01/08–26/08	26	35	4924	2780	550

Table 3
Results of the two-way ANOVA and growth related parameters (mean ± S.E.) in the early harvest (after 30 days of O_3 exposure inside the OTC chambers)

	Green biomass (g dw)	Senescent biomass (g dw)	Flower no.	Root biomass (g dw)	Senescent/ green biomass ratio	Aerial/ subterranean biomass ratio
O_3	<0.0001	0.002	ns	<0.0001	<0.0001	ns
Nitrogen	<0.0001	ns	<0.01	<0.0001	<0.001	ns
O_3 × nitrogen	0.01	ns	ns	ns	ns	ns
CFA low N	0.35±0.04^{a}	0.10±0.01	0	0.19±0.02	0.31±0.03	2.51±0.27
NFA low N	0.34±0.03^{a}	0.11±0.01	2.44±1.53	0.17±0.02	0.35±0.04	3.09±0.35
NFA+ low N	0.24±0.04^{a}	0.11±0.01	0.11±0.10	0.12±0.02	0.58±0.14	3.28±0.29
CFA medium N	0.70±0.07^{b}	0.08±0.01	0.44±0.31	0.27±0.04	0.12±0.02	3.26±0.49
NFA medium N	0.37±0.05^{a}	0.10±0.01	2.88±1.05	0.22±0.02	030±0.04	2.42±0.22
NFA+ medium N	0.33±0.04^{a}	0.14±0.02	0.33±0.31	0.15±0.03	0.48±0.06	3.59±0.36
CFA high N	0.73±0.05^{b}	0.08±0.00	5.33±2.19	0.32±0.02	0.12±0.01	3.01±0.30
NFA high N	0.59±0.03^{ab}	0.12±0.01	2.56±1.05	0.25±0.03	0.20±0.03	3.49±0.41
NFA+ High N	0.43±0.04^{b}	0.13±0.02	3.66±1.32	0.21±0.02	0.32±0.05	2.96±0.10

CFA, charcoal filtered air; NFA, non-filtered air; NFA+, non-filtered air+40 ppb O_3. Different letters indicate significant differences among means in each nitrogen treatment when interactive effects were significant.

Table 4
Results of the two-way ANOVA and growth related parameters (mean ± S.E.) in the late harvest (30 days of exposure to the open after one month of O_3 exposure inside the OTC chambers)

	Total aerial biomass (g dw)	Root biomass (g dw)	Aerial/subterranean biomass ratio	Flower/total aerial biomass ratio	Seed/total aerial biomass ratio
O_3	<0.0001	<0.0001	<0.01	<0.0001	<0.01
Nitrogen	<0.0001	<0.001	ns	ns	<0.01
O_3 × nitrogen	0.05	ns	ns	ns	ns ($p<0.1$)
CFA low N	2.29±0.32^{b}	0.37±0.04	6.21±0.73	0.44±0.03	0.18±0.02
NFA low N	1.73±0.22^{b}	0.34±0.06	5.67±0.50	0.34±0.05	0.13±0.02
NFA+ low N	0.98±0.08^{a}	0.16±0.02	6.29±0.38	0.35±0.02	0.15±0.01
CFA medium N	3.03±0.38^{b}	0.36±0.04	8.56±0.67	0.49±0.02	0.21±0.02
NFA medium N	2.34±0.18^{b}	0.33±0.05	7.80±0.60	0.44±0.03	0.20±0.03
NFA+ medium N	0.81±0.08^{a}	0.19±0.03	5.66±0.97	0.26±0.05	0.10±0.02
CFA high N	4.67±0.49^{b}	0.63±0.07	7.86±0.67	0.36±0.05	0.12±0.02
NFA high N	3.20±0.34^{b}	0.48±0.05	6.82±0.49	0.39±0.03	0.13±0.03
NFA+ high N	1.13±0.13^{a}	0.22±0.02	5.69±0.99	0.23±0.04	0.07±0.02

CFA, charcoal filtered air; NFA, non-filtered air; NFA+, non-filtered air+40 ppb O_3. Different letters indicate significant differences among means in each nitrogen treatment when interactive effects were significant.

et al., 2005). These results indicate the high O_3 sensitivity of this species since an O_3 exposure below the O_3 critical level (1196 nl l^{-1} h in NFA) altered the above-ground biomass reducing green parts while increasing senescent biomass. This high O_3 sensitivity has been already described in previous works where *T. striatum* was considered as the most O_3 sensitive of some 20 species characteristic of therophytic Mediterranean pastures based on the rapid development of foliar symptoms with an accumulated exposure expressed as AOT40 index of only 17 nl l^{-1} h (Bermejo et al., 2003). In that experiment, similar reductions of green biomass were observed but induced by about a three times lower accumulated O_3 exposure (Gimeno et al., 2004a).

Both green and total aerial biomass were also significantly affected by N supplementation (Table 3) but N-induced opposite effects compared to O_3. The total aerial biomass was the highest when N supplement of 30 kg ha^{-1} was applied (half of the total dose of high N treatment), while a 23% and 44% lower biomass production was found when medium (15 kg ha^{-1}) and low (5 kg ha^{-1}) N doses were applied, respectively (average values of all O_3 treatments). Nitrogen did not affect the senescent biomass of *T. striatum*.

An interesting significant N × O_3 interaction was found when considering the green or total biomass yield at the early harvest (Table 3; Fig. 1a). Under low N supplies, N was the main limitation for *T. striatum* growth and no differences induced by O_3 exposure were found. When increasing N supply, O_3 exposure became a modulating factor: the medium N

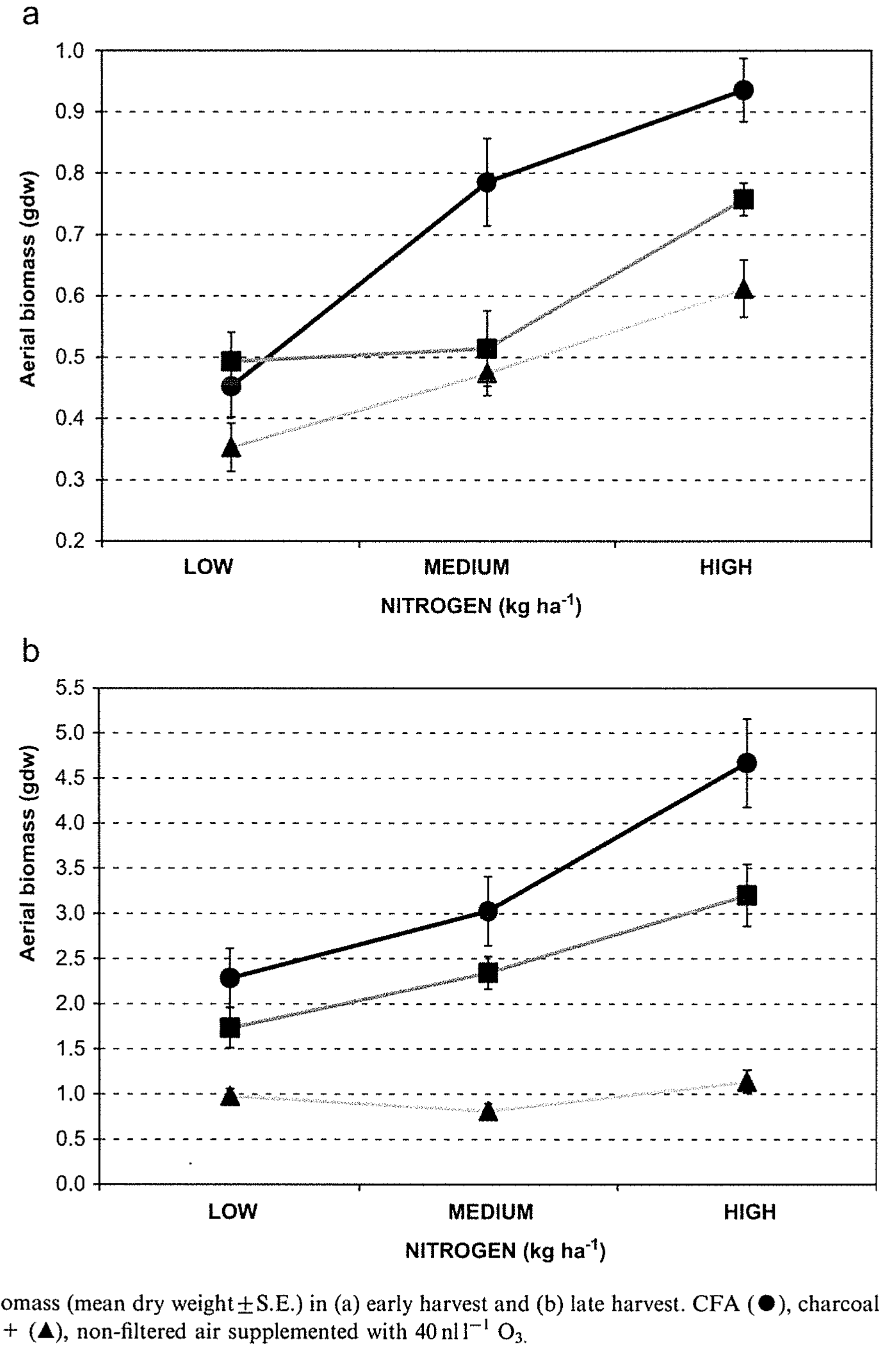

Fig. 1. Total aerial biomass (mean dry weight ± S.E.) in (a) early harvest and (b) late harvest. CFA (●), charcoal filtered air; NFA (■), non-filtered air; NFA + (▲), non-filtered air supplemented with 40 nl l^{-1} O_3.

supplementation caused a stimulation of biomass production only in CFA chambers while the biomass in NFA or NFA+ treatments were 37% lower than in CFA control plants (Fig. 1a). The addition of $30\,\mathrm{kg\,N\,ha^{-1}}$ (two doses of the high N treatment) partly balanced the adverse effects induced by O_3 ambient exposure since no differences were found between NFA and CFA treatments. However, the NFA+ plants showed a 36% reduction of the aerial biomass compared to CFA plants, a percentage close to that observed in the medium N treatment.

3.2.2. *Late harvest*

The late harvest was carried out about 1 month later than the early harvest when plants were already exposed to the open for about 1 month. Early O_3 exposure accelerated plant senescence and shortened life cycle as the seeds of exposed plants achieved ripeness earlier than control plants. Therefore, in order to harvest plant material at the same phenological stage of seed maturity, the second harvest was performed at different times for the different O_3 treatments (Table 1).

Similar O_3 and N effects than in the first period were observed in the biomass of the second harvest but the interactive $O_3 \times N$ effects showed a slightly different pattern (Table 4; Fig. 1b). Ozone induced a total biomass reduction in the NFA treatment of 27% compared to the control CFA (mean values of all N treatments) and up to 75% in NFA+. The greater intensity of the O_3 effect during the second harvest period must be attributed basically to the early exposure inside the chambers and thus it can be considered as a carry-over effect. This kind of responses were also found by Gimeno et al. (2004a) when analyzing O_3 effects on flower production of annual clover species. The greater intensity of the O_3 effect was due to the different growth rates of plants during the period between both harvests. While the aerial biomass production of the CFA plants was four times higher in the second harvest compared to the first one, the NFA+ plants only doubled its early weight production. It was during the second period when plants needed all their resources to stimulate growth and specially flower and seed production; therefore damages suffered in the metabolism during the previous O_3 exposure would have altered the growth during the second period. Nitrogen enrichments effects were also observed in the second harvest, with 31% and 44% reductions of biomass production recorded in medium and low N treatments, respectively, compared to the high N treatment (mean values of all O_3 treatments). Similarly, significant $O_3 \times N$ interactive effects on total aerial biomass were detected in the late harvest, but in contrast with effects found in the early harvest, differences among O_3 treatments were significant even with the low N dose, showing the NFA+ plants a 51% reduction in biomass production compared to CFA and NFA (Fig. 1b). This effect observed at low N level, was intensified with increasing N supplementation until the highest N dose (high N treatment) where the biomass of NFA+ was only one-third of the control treatment value.

The overall $O_3 \times N$ interactive response on aerial biomass was complex and depended on both N dose and O_3 concentration. Nitrogen was the strongest limiting factor at low N doses avoiding any significant response to O_3 treatments at the early harvest. At higher N doses, an interaction between both factors was observed. When plants were exposed to high O_3 levels (NFA+), N enrichment counterbalanced O_3 effects at the first harvest. However, high O_3 levels during the early period prevented almost any growth during the second independently of the N treatment, despite O_3 exposure was reduced to ambient levels during this second period. As a result, N enrichment could not compensate O_3-induced effects on aerial biomass when plants were exposed to high O_3 levels. On the other hand, under moderate O_3 concentrations (NFA), N supplementation modulated O_3 effects counteracting the O_3-induced reductions on total aerial biomass.

A simultaneous study performed with *T. subterraneum*, another annual clover frequently present in the same plant community, also showed the complexity of the $O_3 \times N$ interactions on annual pasture species (Sanz et al., 2005) since the response depended on the parameter analyzed. Ozone and N supplementation alone affected the above ground biomass but no significant interaction was detected between both factors. However, N supplementation ameliorated the O_3-induced increase in senescent/green biomass ratio while enhancing foliar fiber content and therefore reducing the nutritive quality of the forage.

3.3. *Root biomass*

Ozone exposure in OTCs resulted in a 19% and 38% root growth reduction in NFA and NFA+ treatments, respectively, when compared to CFA (values are mean of all N treatments; Table 3). Ozone effects on belowground biomass were still detected in the second harvest, about 30 days after the exposure in OTCs, when root biomass was similar in NFA and control plants but NFA+ showed a 54% reduction compared to CFA (Table 4).

Nitrogen enrichment increased root biomass in early harvest causing an 18% and 39% reduction in medium and low N treatments, respectively, when compared to the highest N dose plants. The addition of the last two N doses until the late harvest produced a final 34% root biomass reduction in medium and low N compared to high N treatment. No significant $O_3 \times N$ interactions were detected in any of the analyzed harvests.

The effects of both O_3 and N enrichment in root biomass were in the range of the effects detected on aerial biomass resulting in no changes on aerial/subterranean biomass ratio in the first harvest. However, a 22% decrease in this ratio was observed in NFA+ plants in the second period compared to control plants due to the stronger O_3 effect on aerial biomass than on belowground at this time.

Similar O_3 effects on root growth of subterranean clover have been reported by Sanz et al. (2005) but in this case, ambient O_3 exposure increased the shoot/root ratio due to a greater O_3 effect on roots compared to above ground biomass. Both increases and reductions of the aerial/root ratio have been described in different Leguminosae annual species of this community (Gimeno et al., 2004a). Alteration of carbon allocation is a common O_3 effect that is species dependent and might affect competitiveness ability and plant community composition (Franzaring et al., 2000).

3.4. *Reproductive ability*

The first analysis of floral production was completed at the time of the early harvest. No O_3 effect was detected at this stage but the highest N supplement resulted in a greater number of developing flowers per plant (Table 3). During this period of plant vegetative growth, O_3 exposure hastened plant senescence and seed ripening. Seed ripening first occurred in the NFA+ treatment; 56 days after the start of the experiment (DSE); followed by NFA (63 DSE) and CFA (67 DSE) plants. The premature senescence induced by O_3 has been widely reported in the literature (Grandjean and Fuhrer, 1989; Pleijel et al., 1991).

At the late harvest, O_3 exposure, N input and their interaction induced significant effects on the reproductive ability of *T. striatum* (Table 4). Ozone carry-over effects caused reductions of flower (Fig. 2a) and seed (Fig. 2b) biomass by 30% and 80% in NFA and NFA+ treatments, respectively, compared to CFA plants ($p < 0.0001$). This response could potentially affect the structure and diversity of Mediterranean terophytic pastures since their regeneration is greatly dependent on the seed-bank and takes place following the first autumn rainfall (Peco et al., 1998b). An accumulated O_3 exposure of AOT40 = 1795 $\text{nl}\,\text{l}^{-1}\,\text{h}$ for 2 months reduced seed output of *T. striatum*. This value is lower than the previously reported by Gimeno et al. (2004b) where a 29% reduction in the seed output of this species was found following an exposure of AOT40 = 2403 $\text{nl}\,\text{l}^{-1}\,\text{h}$. Both values are within the range of the critical level of O_3 for the protection of semi-natural vegetation (UNECE, 2004).

On the contrary, N enrichment increased flower biomass with the greatest biomass recorded in those plants supplemented with the highest N dose, showing increments of 15% and 36% over the plants supplemented with medium or low N doses, respectively ($p < 0.01$). A significant $O_3 \times N$ interaction ($p \leqslant 0.01$) was detected for both parameters related to plant reproductive ability since the application of medium (30 kg N ha^{-1}) or high (60 kg N ha^{-1}) N doses compensated the adverse effects induced by O_3 on flower biomass and seed weight under moderate O_3 levels (NFA treatment) but not when plants were exposed to high O_3 concentrations (NFA+). Seed weight and flower biomass were 82–84% lower in NFA+ compared to CFA or NFA in those plants supplemented with medium (30 kg N ha^{-1}) or high (60 kg N ha^{-1}) when compared to CFA or NFA (Fig. 2). According to the results, N input modulated the severity of the adverse effects induced by moderate O_3 exposures on the reproductive ability of *T. striatum*.

Interestingly, the previous exposure to high O_3 levels during the vegetative growth period of the plants affected later reproductive ability and seed output regardless N treatment. This is an important result since the highest O_3 concentrations in this area occur in spring, at the time of maximum vegetative growth in annual plants. Later in the season, high temperatures and drought would prevent the absorption of the pollutant while inducing annual plants senescence.

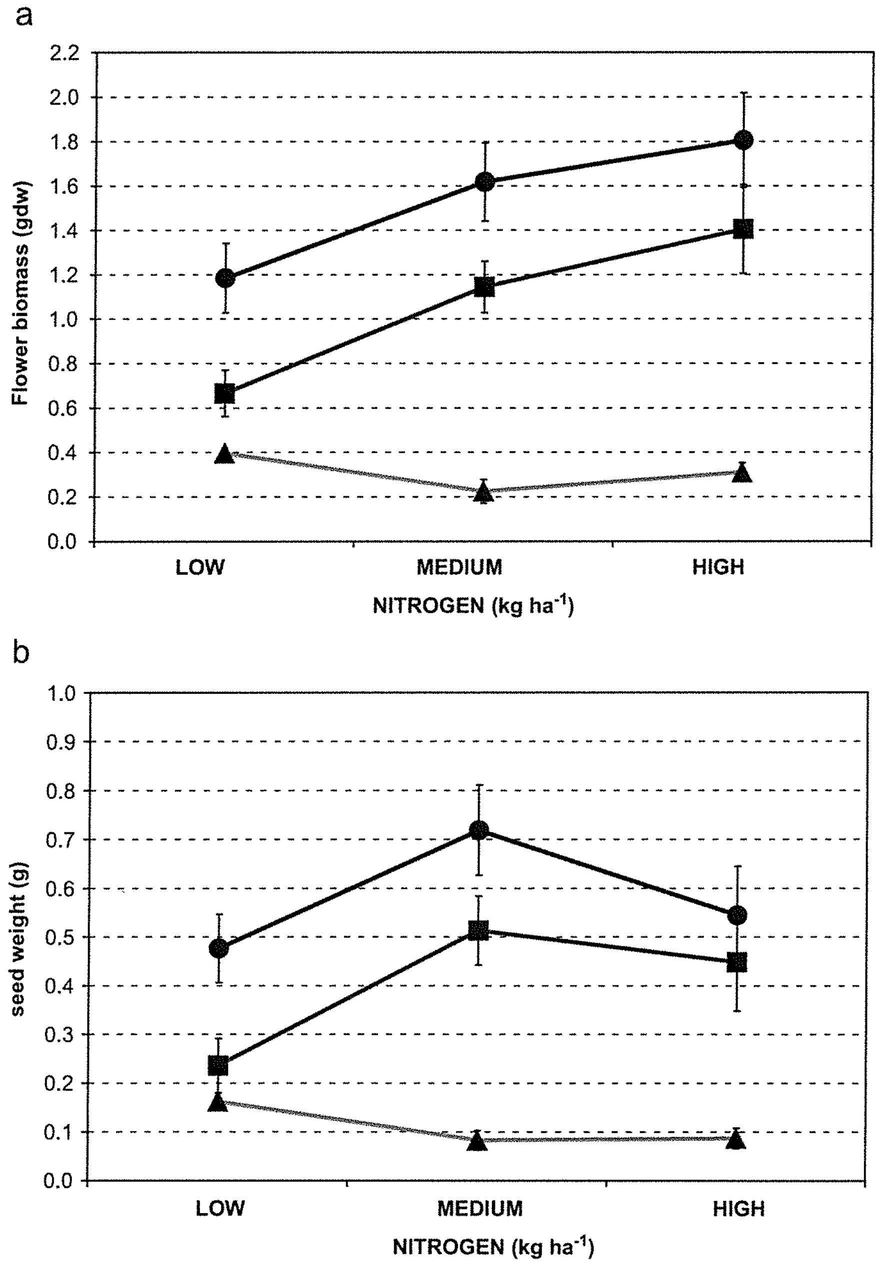

Fig. 2. Reproductive ability expressed as (a) plant flower biomass and (b) plant seed weight. Values are mean dry weight ± S.E. CFA (●), charcoal filtered air; NFA (■), non-filtered air; NFA+ (▲), non-filtered air supplemented with 40 nl l^{-1} O_3.

4. Conclusions

T. striatum can be considered as a very sensitive species to O_3 under experimental conditions regarding both biomass production and reproductive ability. Ozone affected clover phenology as well by shortening plant life span and causing premature senescence. Recent reviews on O_3 sensitivity of semi-natural vegetation pointed out the high sensitivity of therophytes and legumes to high O_3 concentrations (Bassin et al., 2007; Hayes et al., 2007). Ozone-induced effects in *T. striatum* were also detected 30 days after O_3 exposure has ceased and can be considered as carry-over effects. The results indicate that high O_3 levels during the vegetative growth period of annual plants clearly affected flower and seed production and therefore species viability. Effects of O_3 on plant and flower biomass and seed production were partially counterbalanced by soil N enrichment only when plants were exposed to moderate O_3 levels but not when plants were exposed to high O_3 concentrations. These effects could potentially have implications for the structure and diversity of dehesa grasslands if future research indicates that the observed effects also occur under natural conditions.

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References

Allué Andrade, J.L., Tella, G., 1986. Los Pastos Españoles. II. Clasificación, Composición, Atributos y Mejora de sus Principales Tipos Florísticos. E.U.I.T. Forestal, Madrid, Spain.

Alonso, R., Elvira, S., Castillo, F.J., Gimeno, B.S., 2001. Interactive effects of ozone and drought stress on pigments and activities of oxidative enzymes in *Pinus halepensis*. Plant Cell and Environment 24, 905–916.

Bassin, S., Volk, M., Fuhrer, J., 2007. Factors affecting the ozone sensitivity of temperate European grasslands: an overview. Environmental Pollution 146, 678–691.

Bermejo, V., Gimeno, B.S., Sanz, J., de la Torre, D., Gil, J.M., 2003. Assessment of the ozone sensitivity of 22 native plant species from Mediterranean annual pastures based on visible injury. Atmospheric Environment 37, 4667–4677.

Elvira, S., Alonso, R., Castillo, F.J., Gimeno, B.S., 1998. On the response of pigments and antioxidants of *Pinus halepensis* seedlings to Mediterranean climatic factors and long-term ozone exposure. New Phytologist 138, 419–432.

Fenn, M.E., Poth, M., Bytnerowicz, A., Sickman, J.O., Takemoto, B.K., 2003. Effects of ozone, nitrogen deposition and other stressors on montane ecosystems in the Sierra Nevada. In: Bytnerowicz, A., Arbaugh, M., Alonso, R. (Eds.), Ozone Air Pollution in the Sierra Nevada: Distribution an Effects on Forests. Elsevier, UK, pp. 111–157.

Franzaring, J., Tonneick, A.E., Kooijman, A.W., Dueck, T.A., 2000. Growth responses to ozone in plant species from wetlands. Environmental and Experimental Botany 44 (1), 39–48.

Fuhrer, J., Booker, F., 2003. Ecological issues of ozone: agricultural issues. Environment International 29, 141–154.

Fumagalli, I., Gimeno, BS., Velissariou, D., Mills, G., De Temmerman, L., Fuhrer, J., 2001. Evidence of ozone-induced adverse effects on Mediterranean vegetation. Atmospheric Environment 35, 2583–2587.

Gimeno, B.S., Bermejo, V., Sanz, J., de la Torre, D., Elvira, S., 2004a. Growth response to ozone of annual species from Mediterranean pastures. Environmental Pollution 132, 297–306.

Gimeno, B.S., Bermejo, V., Sanz, J., de la Torre, D., Gil, J.M., 2004b. Assessment of the effects of ozone exposure and plant competition on the reproductive ability of three therophytic clover species from Iberian pastures. Atmospheric Environment 38, 2295–2303.

Grandjean, A., Fuhrer, J., 1989. Growth and leaf senescence in spring wheat (*Triticum aestivum* cv. Albis) grown at different ozone concentrations in open-top field chambers. Physiologia Plantarum 77, 389–394.

Hayes, F., Jones, M.L.M., Mills, G., Ashmore, M., 2007. Meta-analysis of the relative sensitivity of semi-natural vegetation species to ozone. Environmental Pollution 146, 754–762.

Krupa, S., Muntifering, R., Chappelka, A., 2004. Effects of ozone on plant nutritive quality characteristics for ruminant animals. The Botanica 54, 1–12.

Millán, M.M., Mantilla, E., Salvador, R., Carratalá, A., Sanz, M.J., Alonso, L., Gangoiti, G., Navazo, M., 2000. Ozone cycles in the western Mediterranean basin: interpretation of monitoring data in complex coastal terrain. Journal of Applied Meteorology 39, 487–508.

Mills, G., Ball, G., Hayes, F., Fuhrer, J., Skärby, L., Gimeno, B.S., De Temmerman, L., Heagle, A.S., 2000. Development of a multi-factor model for predicting the critical level of ozone for biomass reduction for white clover. Environmental Pollution 109, 533–542.

Montoya, J.M., Mesón, M.L., Ruiz del Castillo, J., 1988. Una dehesa testigo: La dehesa de Moncalvillo. Serie Técnica No. 5, Ministerio de Agricultura Pesca y Alimentación, Madrid, Spain.

Nebel, B., Fuhrer, J., 1994. Inter- and intra-specific differences in ozone sensitivity in semi-natural plant communities. Angewandte Botanik 68, 116–121.

Palacios, M., Kirchner, F., Martilli, A., Clappier, A., Martín, F., Rodríguez, M.E., 2002. Summer ozone episodes in the Greater Madrid area. Analysing the ozone response to abatement strategies by modeling. Atmospheric Environment 36, 5323–5333.

Peco, B., Espigares, T., Levassor, C., 1998a. Trends and fluctuations in species abundance and richness in Mediterranean annual pastures. Applied Vegetation Science 1, 21–28.

Peco, B., Ortega, M., Levassor, C.M., 1998b. Similarity between seed bank and vegetation in Mediterranean grasslands: a predictive model. Journal of Vegetation Science 9, 815–828.

Plaza, J., Pujadas, M., Artíñano, B., 1997. Formation and transport of the Madrid ozone plume. Journal of Air and Waste Management Association 47, 666–774.

Pleijel, H., Skärby, L., Wallin, G., Sellden, G., 1991. yield and grain quality of spring wheat (*Triticum aestivum* L.; Cv. Drabant) exposed to different concentrations of ozone in open-top chambers. Environmental Pollution 69, 151–168.

Pujadas, M., Plaza, J., Terés, J., Artíñano, B., Millán, M., 2000. Passive remote sensing of nitrogen dioxide as a tool for tracking air pollution in urban areas: the Madrid urban plume, a case of study. Atmospheric Environment 34, 3041–3056.

Rodà, F., Avila, A., Rodrigo, A., 2002. Nitrogen deposition in Mediterranean forests. Environmental Pollution 118, 205–213.

Sala, O.E., Chapin III, F.J., Armesto, J.J., Berlow, E., Bloomfield, J., Dirzo, R., Huber-Sanwald, E., Huenneke, L., Jackson, R.B., Kinzig, A., Leemans, R., Lodge, D., Mooney, H., Oesterheld, M., Poff, N.L., Sykes, M.T., Walker, B.H., Walker, M., Wall, D.H., 2000. Global diversity scenarios for the year 2100. Science 287, 1770–1774.

Sanz, J., Muntifering, R.B., Bermejo, V., Gimeno, B.S., Elvira, S., 2005. Ozone and increased nitrogen supply effects on the yield and nutritive quality of *Trifolium subterraneum*. Atmospheric Environment 39, 5899–5907.

Takemoto, B.K., Bytnerowicz, A., Fenn, M.E., 2001. Current and future effects of ozone and atmospheric nitrogen deposition on California's mixed conifer forest. Forest Ecology and Management 144, 159–173.

The Fertilizer Institute, 1982. The Fertilizer Handbook. Washington, DC, 2002.

UNECE, 2004. Manual on methodologies and criteria for modelling and mapping critical loads and levels and air pollution effects, risks and trends. Convention on Long-range Transboundary Air Pollution. ⟨http://www.icpmapping.org⟩.

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Plant phenology, growth and nutritive quality of *Briza maxima*: Responses induced by enhanced ozone atmospheric levels and nitrogen enrichment

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Forage quality and phenology are more O_3-sensitive than growth parameters in the Mediterranean annual grass Briza maxima.

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ABSTRACT

An assessment of the effects of tropospheric ozone (O_3) levels and substrate nitrogen (N) supplementation, singly and in combination, on phenology, growth and nutritive quality of *Briza maxima* was carried out. Two serial experiments were developed in Open-Top Chambers (OTC) using three O_3 and three N levels. Increased O_3 exposure did not affect the biomass-related parameters, but enhanced senescence, increased fiber foliar content (especially lignin concentration) and reduced plant life span; these effects were related to senescence acceleration induced by the pollutant. Added N increased plant biomass production and improved nutritive quality by decreasing foliar fiber concentration. Interestingly, the effects of N supplementation depended on meteorological conditions and plant physiological activity. N supplementation counteracted the O_3-induced senescence but did not modify the effects on nutritive quality. Nutritive quality and phenology should be considered in new definitions of the O_3 limits for the protection of herbaceous vegetation.

1. Introduction

Elevated tropospheric ozone (O_3) levels and nitrogen (N) enrichment of ecosystems are two of the main components of global change, induced by the increase of human activity from the start of the industrial times. The current O_3 background levels have doubled the values found in the last century and keep rising at an annual rate of 0.5–2% (Vingarzan, 2004). The legal restrictions imposed in some countries to control its precursors' emissions have been effective in decreasing episodic peak O_3 levels (Derwent et al., 2003), but no effects on background levels have been observed; in fact, tropospheric O_3 concentration is still increasing (Jonson et al., 2006). Mediterranean areas are among the European Union (EU) areas where higher O_3 levels are recorded, as influenced by the emission of O_3 precursors and climatic conditions (elevated solar radiation, scarce rainfall, stagnation of polluted air masses) that favour O_3 yield from photochemical reactions, especially in spring and summer (Nolle et al., 2002). Several authors (Langner et al., 2005; Meleux et al., 2007) have consistently agreed that, under the climate change scenarios considered by the Intergovernmental Panel on Climate Change (IPCC), O_3 concentrations in the Mediterranean areas will rise during the current century as a result of warmer and drier summers, and the reduction of cloud cover.

Tropospheric O_3 is considered nowadays as the most phytotoxic atmospheric pollutant. Ozone levels high enough to produce detrimental effects on Mediterranean sensitive plant species have been registered in many studies carried out under field and semi-controlled conditions (Gimeno et al., 1999; Ribas and Peñuelas, 2003; Nali et al., 2006; Ferretti et al., 2007; Calvo et al., 2007).

The global N cycle has also been profoundly altered by anthropogenic activities that had doubled the transfer from the immense and unreactive atmospheric N pool to the biologically available forms (Vitousek et al., 1997; Gruber and Galloway, 2008). The increased use of fossil fuel combustion for transport and industrial processes has been the main source of oxidised N to the atmosphere; whereas the evolution towards more intensive and productive cattle and agriculture systems has been responsible for the release of reduced N compounds. When N deposition overloads N assimilation capacity of the ecosystems, changes occur in plant susceptibility to other biotic or abiotic factors (Jones et al., 2008), nutrient imbalances (Elvir et al., 2006), alteration of species phenology (Cleland et al., 2006) or changes in plant community composition and structure (Van Der Wal et al., 2003; Bobbink et al.,

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2010). The estimates of total N deposition in the Mediterranean area are in the range of 10–38 kg N $ha^{-1}year^{-1}$ (Sanz et al., 2002; Rodà et al., 2002), a relatively high value considering that slight increases in atmospheric deposition, around 5 kg N ha^{-1} $year^{-1}$, are enough to produce changes in ecosystem flora (Fenn et al., 2008). Nitrogen enrichment is considered as one of the main driving forces in altering Mediterranean terrestrial ecosystems (Sala et al., 2000).

The coexistence of high levels of O_3 and high N deposition on the same area is considered as the most harmful combination of environmental stresses affecting forest communities (Fowler et al., 1999). This coincidence has been described in the Dehesa ecosystems north of Madrid City in central Spain (Plaza et al., 1997; Alonso et al., 2009). Nitrogen enrichment reaches the system not only via atmospheric transport from Madrid City but also from extensive cattle whose manure produces important but scattered N inputs.

The dehesa ecosystem, an open oak forest with an annual pasture understorey, is a valuable example of sustainable management of natural resources where agricultural, timber and extensive livestock exploitation coexist over an area of 3.5–4 million hectares in the Iberian Peninsula (Olea and San Miguel-Ayanz, 2006). Dehesa formations have high species richness (Peco et al., 1998), and are presently protected by the 92/43/EEC Habitat Directive and included in the Nature 2000 network. Efforts have been developed recently aiming to understand the O_3 sensitivity of dehesa therophytes (Bermejo et al., 2003; Gimeno et al., 2004a,b), and how N modulates their response to O_3 (Sanz et al., 2005, 2007). These experimental results, based on foliar symptoms, biomass production and reproductive ability, have shown a greater O_3 sensitivity of *Leguminoseae* compared with *Gramineae* species, and complex O_3 * N interactions with contrasting results depending on the plant parameter considered. However, despite its implications for livestock feeding (Krupa et al., 2004), studies about the combined effects of O_3 and N on the nutritive quality of the dehesa forage are scarce and only some O_3-sensitive legumes have been assayed (Sanz et al., 2007).

The present study involved the annual grass species *Briza maxima*, one of the most characteristic species of the oligotrophic Mediterranean pastures (Perez Prieto and Font, 2005), which has been previously classified as being relatively resistant to O_3 based on its biomass and foliar injury responses to this pollutant (Bermejo et al., 2003; Gimeno et al., 2004a). The hypothesis was that N sensitized the response of *B. maxima* to O_3 making the species more susceptible to O_3 damage considering both quality and yield parameters.

2. Materials and methods

Experimental work was developed in an open-top chamber facility (OTC) described in Alonso et al. (2001), located in a rural area far from major sources of pollutants (40°41′N, 0°47′E).

2.1. Plant material and treatments

B. maxima seeds were collected in the Dehesa of Moncalvillo (Guadalix de la Sierra, Madrid, 40°40′N 03°46′W) and sown in trays using a 50% neutral peat and 50% vermiculite substrate. Seedlings were transplanted to 2.5-L pots with a 50% peat, 30% vermiculite and 20% perlite substrate; pH was adjusted to neutrality by adding CaO. A low-N fertilizer (Peters, NPK: 4/25/35 plus microelements) was provided to prevent nutritional deficiencies. Four additional applications of different doses of NH_4NO_3 solution were performed every 15 days in order to achieve N integrated doses at the end of the experiment of 10, 30 and 60 kg ha^{-1} corresponding to the Low (Low-N), Medium (Medium-N) and High (High-N) nitrogen treatments, respectively (Table 1). These N integrated doses were selected to reflect the natural variability found in N inputs in the Dehesa soils of the Iberian Peninsula, considering both N entering via manure (normal stocking rate is less than one cow ha^{-1}) and expected atmospheric N deposition reported by Rodà et al. (2002) in broadleaf evergreen forests in NE Spain (15–22 g N deposited ha^{-1} y^{-1}).

Table 1
Timetable of events for the *Briza* assays.

	Event	Date	DaS
First assay	Sowing	27 May	
	Emergence	29 May	
	N fertilization	29 May	
	Transplantation	1 July	
	Start of ozone exposure in OTCs	2 July	0
	N fertilization	15 July	13
	N fertilization (second set of plants)	29 July	27
	Harvest	7–9 August	36–38
	First phenology assay	9 August	38
	End of OTC, exposure to open	12 August	41
	N fertilization (second set of plants)	21 August	50
	Second phenology assay	30 August	59
	Third phenology assay in NFA+	12 September	72
	Third phenology assay in NFA	18 September	78
	Third phenology assay in CFA	25 September	85
Second Assay	Sowing	19 September	
	Transplantation	30 September	
	Start of ozone exposure in OTCs	1 October	0
	N fertilization	2 October	1
	N fertilization	21 October	20
	N fertilization	8 November	39
	End of OTC exposure	12 November	43
	Harvest	14 November	45

DaS (Days after start of experiment).

Two serial assays were performed using the same experimental design, schedules for which are presented in Table 1. *B. maxima* seedlings were exposed during one month to three O_3 treatments: charcoal-filtered air (CFA), non-filtered air (NFA) or non-filtered air supplemented with 40 nl l^{-1} O_3 from 07:00 to 17:00 (GMT) 5 days $week^{-1}$ (NFA+). Ozone concentrations in the NFA+ treatment were in the range of those normally experienced by this species under field conditions (Alonso et al., 2009). An automatic system provided continuous monitoring of O_3, sulphur dioxide and NO_x concentrations in the different treatments. Three OTC replicates per O_3 treatment were used. Sets of 90 plants were used in each assay, to allow 9 homogeneous plants per O_3 exposure and N treatment combination leaving some extra plants in case of any lost. During the first assay, two sets were employed, one to determine biomass-related parameters immediately after the exposure period in the OTCs, and the other for the assay of flower and seed production at the end of plant life span. To conduct the latter analyses, plants were transferred to the open after O_3 exposure until they had completed their life span and seeds were formed. A drip irrigation system was used throughout both experiments to ensure adequate and homogeneous water availability.

2.2. Charaterization of ozone exposure and meteorological conditions

Mean hourly O_3 profiles were calculated for the different O_3 treatments and assays. The value of the AOT40 index was calculated as it is currently used by both European legislation (2008/50/EC) and the Convention of Long Range Transboundary Air Pollution of the United Nations – Economic Commission for Europe (UN/ECE, 2008) to assess the phytotoxical risk derived from O_3 exposure. Data of wind speed and direction, air temperature (T) and relative humidity (RH), and global radiation were obtained from the closest station of the official network of the Catalan Meteorological Service (Spain).

2.3. Biomass production

Aerial and root biomass harvests were performed after 30 days of O_3 exposure. The aerial biomass production was obtained by cutting the plants at the surface of the substrate; roots were also carefully extracted and washed, and all samples were dried at 60°C until constant weight was reached. Aerial biomass was separated into green and senescent leaves to estimate green/senescent ratio. Additionally, in the first experiment, fresh weight was determined to enable calculation of fresh/dry aerial biomass ratio. Harvesting dates are presented in Table 1.

2.4. Nutritive quality parameters

Nutritive quality parameters were analysed using the samples harvested during the first experiment. Composite samples were generated by pooling the dried green biomass of three plants per treatment. Dry matter (DM) was determined by drying samples at 100 °C to constant weight, and concentration of crude protein ($CP = \% N \times 6.25$) was determined by the Kjeldhal method (Association of Official Analytical Chemists, 1995). Forage concentrations of neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined sequentially according to procedures of Van Soest et al. (1991). Relative feed value (RFV) was calculated on the basis of foliar concentrations of NDF and ADF (Linn and Martin, 1989) to derive a single index of nutritive quality of *Briza* forage. Chemical analyses were conducted in the Department of Animal Sciences at Auburn University (USA).

2.5. Plant phenology

Plant phenology was assessed throughout the first experiment (Table 1). Plants were classified each sampling date according to the following phenological stages: 1. Vegetative stage, ears not formed; 2. Start of flowering, ear stems growing although ears were not yet developed; 3. Ear developing stage, plants with developing ears and greenish panicles; 4. Seed ripening, plants with mature ears and dry/yellowish panicles indicating seed maturity close to anemochoric dispersion. The evaluations were carried out at 38, 60, and 70 days after the start of O_3 exposure (DaS).

2.6. Statistical analysis

The effects of N fertilization and O_3 exposure on the different parameters were evaluated by performing independent two-way ANOVA analyses. When significant differences among treatments were detected ($p < 0.05$), mean differences were assessed using the Tukey honest significant difference test. Normal probability plots and scatter plots of residuals were used to test data normality. Levenne test was applied to check variance homoscedasticity. When non-compliance with ANOVA assumptions was observed, data were transformed to fulfill the statistical requirements. All statistical analyses were carried out using Statistica 5.1 software (StatSoft, Inc., USA).

3. Results

3.1. Meteorological conditions and ozone levels

High RH values in the range of 70–90% were recorded in both assays but 5–7 °C higher T were registered in the first assay resulting in noticeable higher VPD values compared with the second assay (Fig. 1). Solar radiation was also more elevated during the first assay, not only in regards of accumulated light hours but also regarding solar radiation intensity.

Ozone values registered during the assays are within the seasonal and inter-annual variability of O_3 levels usually found in the dehesa pastures where *B. maxima* widely occurs. Ambient O_3 levels (NFA) were higher during the first assay performed in spring-summer compared with the second experiment carried out in fall (Fig. 2). AOT40 in the NFA treatment was 1367 nl l^{-1} h (37 days) and 221 nl l^{-1} h (45 days) for the first and second assay, respectively. Similarly, the AOT40 value of the NFA+ treatment was 10,841 nl l^{-1} h in the first experiment, but it was 44% lower (6032 nl l^{-1} h) in the second assay. Ozone levels in the control treatment (CFA) were always below 10 nl l^{-1}; therefore, the AOT40 value of this treatment was zero in both experiments.

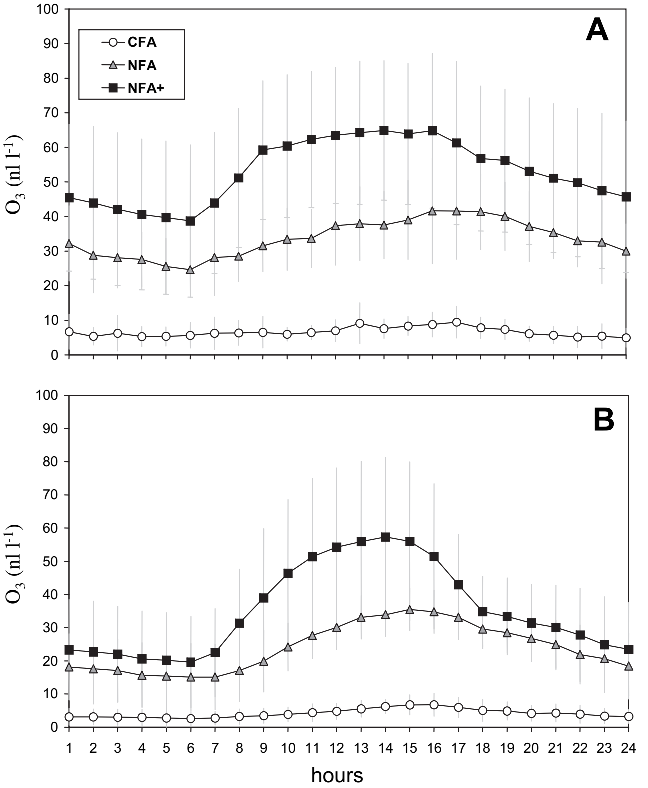

Fig. 2. Ozone daily profiles for *Briza* assays inside the OTC (mean ± sd) for the first (A) and second (B) assays.

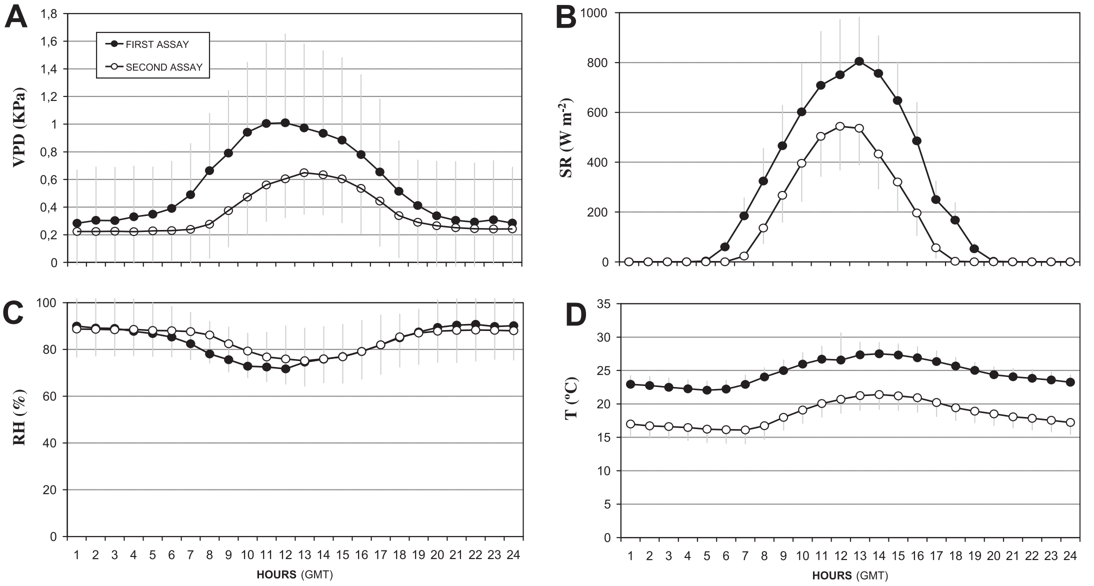

Fig. 1. Mean daily profiles of meteorological conditions during the *Briza* assays (mean ± sd). A) water vapour pressure deficit (VPD); B) total solar radiation; C) air relative humidity; D) air temperature.

Table 2
Growth-related parameters (means per plant ± se) corresponding to the exposure of *Briza maxima* to the different O_3 and N treatments on the first assay.

First assay	Green biomass (g dw)	Senescent biomass (g dw)	Senes./green biomass Ratio	Total aerial biomass (g dw)	Subt. biomass (g dw)	Total biomass (g dw)	Aerial/Subt. ratio	Fresh aerial biomass (g fw)	Fresh/dry biomass ratio
O_3	ns	<0.001	<0.0001	ns	ns	ns	ns	ns	ns
Nitrogen	<0.0001	<0.005	<0.01	<0.0001	<0.0001	<0.0001	<0.05	<0.0001	ns
*O_3 * Nitrogen*	ns	ns	ns	ns	ns	ns	ns	ns	ns
Low-N CFA	0.73 ± 0.10	0.071 ± 0.01	0.12 ± 0.03	0.80 ± 0.09	0.72 ± 0.05	1.52 ± 0.13	1.10 ± 0.08	5.33 ± 0.67	6.09 ± 0.13
Low-N NFA	0.67 ± 0.04	0.05 ± 0.01	0.08 ± 0.01	0.73 ± 0.04	0.75 ± 0.03	1.48 ± 0.07	0.97 ± 0.03	3.80 ± 0.30	6.07 ± 0.07
Low-N NFA+	0.86 ± 0.11	0.13 ± 0.02	0.15 ± 0.01	1.00 ± 0.13	0.84 ± 0.12	1.84 ± 0.24	1.23 ± 0.08	5.35 ± 0.95	6.01 ± 0.10
Medium-N CFA	1.03 ± 0.08	0.06 ± 0.00	0.06 ± 0.01	1.10 ± 0.08	0.86 ± 0.05	1.95 ± 0.12	1.25 ± 0.04	6.77 ± 0.19	5.82 ± 0.20
Medium-N NFA	1.09 ± 0.08	0.08 ± 0.01	0.07 ± 0.01	1.17 ± 0.09	1.18 ± 0.10	2.36 ± 0.18	0.09 ± 0.03	6.60 ± 0.59	6.16 ± 0.15
Medium-N NFA+	1.18 ± 0.10	0.17 ± 0.02	0.14 ± 0.01	1.35 ± 0.12	1.18 ± 0.14	2.54 ± 0.24	1.21 ± 0.10	7.00 ± 0.79	5.85 ± 0.27
High-N CFA	1.90 ± 0.17	0.09 ± 0.03	0.04 ± 0.01	1.99 ± 0.19	1.38 ± 0.09	3.37 ± 0.25	1.45 ± 0.13	12.50 ± 0.91	5.62 ± 0.07
High-N NFA	1.77 ± 0.14	0.14 ± 0.02	0.08 ± 0.01	1.91 ± 0.14	1.59 ± 0.13	3.51 ± 0.23	1.24 ± 0.11	11.63 ± 0.90	6.22 ± 0.43
High-N NFA+	1.96 ± 0.16	0.21 ± 0.03	0.10 ± 0.01	2.17 ± 0.18	1.62 ± 0.16	3.80 ± 0.31	1.38 ± 0.09	11.60 ± 0.56	5.87 ± 0.08

The *p* values corresponding to the effect of each factor (O_3 and N) are presented in the first part of the table while the values (mean ± se) are presented below. CFA = charcoal filtered air; NFA = non-filtered air; NFA+ = non filtered air + 40 ppb O_3; Low, Medium and High-N represent N supply at 5, 15 and 30 kg ha^{-1}; respectively.

3.2. Biomass production

In the first experiment, aerial senescent biomass per plant was significantly enhanced by O_3 exposure ($p < 0.001$) across all N treatments (Table 2). This increase was the highest (140%) in the NFA+ treatment compared with control (CFA). No O_3 effects were found in aerial green biomass (dry weight); as result, a significant increase of the senescent/green biomass ratio was detected ($p < 0.0001$), with an 85% higher ratio in the NFA+ treatment compared with the control. No O_3 effects were found on total aerial biomass (senescent + green), subterranean biomass, total biomass (aerial + subterranean) or aerial/subterranean biomass ratio. Similarly, no effects were observed on fresh green biomass or fresh/dry green biomass ratio. Therefore, the pollutant did not alter the foliar water content of *B. maxima*.

In the second assay, O_3 exposure induced similar effects to those found in the first experiment, increasing foliar senescence by 120% ($p < 0.001$) in the NFA+ treatment compared with the control (Table 3). A similar trend was observed in the NFA treatment. Senescent/green biomass ratio was also altered ($p < 0.0001$), even though no O_3 effect on green biomass was observed; this ratio increased ca. 150% in NFA+ compared with CFA chambers, slightly higher than the increment recorded during the first assay.

Total aerial biomass, subterranean biomass, total biomass (subterranean + aerial) and aerial/subterranean biomass ratio were not affected by O_3 exposure during the second assay, in agreement with the results from the first experiment.

Substrate N enrichment caused a general stimulation of different growth-related parameters In the first assay, the Medium-N level increased green, total aerial, subterranean, and total biomass by ca. 40% across all O_3 treatments with unaltered aerial/subterranean ratio (Table 2). With the High-N dose, green and total aerial biomass were increased by about 145% across all O_3 treatments compared with the Low-N level but the effect on subterranean biomass was lower (ca. 100%); thus, a 23% increase in the aerial/subterranean biomass ratio was observed (Table 2). Nitrogen supplementation induced a 30% reduction of the senescent/green biomass ratio in the High-N treatment. On the other hand, added N did not alter the fresh/dry biomass ratio.

Although N effects in the second assay were similar in pattern to those in the first experiment, they were smaller and statistically significant only for the highest level of N supplementation (Table 3). In agreement with the first experiment, the High-N level caused 44% and 16% increases of the aerial and subterranean biomass respectively, reproducing the increase of 24% of the aerial/subterranean ratio observed in the first experiment. No N effect on foliar senescence but a significant $O_3 \times N$ interaction on foliar senescence and senescent/green biomass ratio was found. In this sense, the addition of the High N dose counteracted the adverse effects induced by the high O_3 exposure (NFA+) treatment.

Table 3
Growth-related parameters (mean ± se) corresponding to the exposure of *Briza maxima* to the different O_3 and N treatments on the second assay.

Second assay	Green biomass (g dw)	Senescent biomass (g dw)	Senes./green biomass ratio	Total aerial biomass (g dw)	Subt. biomass (g dw)	Total biomass (g dw)	Aerial/Subt. ratio
O_3	ns	<0.001	<0.0001	ns	ns	ns	ns
Nitrogen	<0.0001	ns	<0.01	<0.0001	<0.0001	<0.0001	<0.001
*O_3 * Nitrogen*	ns (0.8)	<0.01	<0.01	ns	ns (0.1)	ns	ns
Low-N CFA	2.87 ± 0.11	0.11 ± 0.01^{a}	0.038 ± 0.005^{a}	2.97 ± 0.11	2.77 ± 0.20	5.75 ± 0.26	1.11 ± 0.07
Low-N NFA	2.82 ± 0.13	0.10 ± 0.01^{a}	0.037 ± 0.004^{a}	2.92 ± 0.13	2.48 ± 0.10	5.41 ± 0.14	1.19 ± 0.08
Low-N NFA+	2.77 ± 0.17	0.20 ± 0.04^{b}	0.080 ± 0.020^{b}	2.97 ± 0.14	2.63 ± 0.09	5.61 ± 0.20	1.13 ± 0.05
Medium-N CFA	3.22 ± 0.11	0.09 ± 0.01^{a}	0.028 ± 0.004^{a}	3.31 ± 0.11	2.87 ± 0.17	6.19 ± 0.21	1.19 ± 0.09
Medium-N NFA	2.79 ± 0.08	0.14 ± 0.02^{a}	0.046 ± 0.005^{a}	3.12 ± 0.09	2.74 ± 0.18	5.86 ± 0.23	1.16 ± 0.07
Medium-N NFA+	2.74 ± 0.10	0.29 ± 0.05^{b}	0.115 ± 0.019^{b}	3.05 ± 0.09	2.49 ± 0.16	5.54 ± 0.22	1.25 ± 0.07
High-N CFA	4.20 ± 0.14	0.11 ± 0.01^{a}	0.025 ± 0.002^{a}	4.31 ± 0.14	2.98 ± 0.11	7.30 ± 0.24	1.44 ± 0.02
High-N NFA	3.85 ± 0.12	0.14 ± 0.02^{a}	0.036 ± 0.004^{a}	4.00 ± 0.12	3.11 ± 0.22	7.11 ± 0.29	1.32 ± 0.08
High-N NFA+	4.27 ± 0.11	0.16 ± 0.01^{a}	0.037 ± 0.002^{a}	4.43 ± 0.12	3.08 ± 0.28	7.46 ± 0.30	1.51 ± 0.14

The *p* values corresponding to the effect of each factor (O_3 y N) are presented in the first part of the table while the values (mean ± se) are presented below. CFA = charcoal filtered air; NFA = non-filtered air; NFA+ = non filtered air + 40 ppb O_3 Low, Medium and High-N represent N supply at 5, 15 and 30 kg ha^{-1}; respectively. Different letters indicate significant differences among O_3 treatments according to the $O_3 \times N$ interaction ($p < 0.05$).

3.3. Nutritive quality

Concentrations of ADF, NDF and lignin were greater following exposure to elevated O_3 across all N levels (Table 4). Foliar fiber concentration of ADF and NDF increased 8-9% and lignin content increased 89% and 121%, respectively, in both NFA and NFA+ treatments compared with control. A trend towards a reduction in crude protein concentration as influenced by O_3 was also observed ($p = 0.05$). In accordance with these results, nutritive quality defined in terms of RFV was decreased around 10% in NFA and NFA+ treatments compared with control.

Nitrogen fertilization improved RFV by 9% N because high-N treated plants contained a 6–7% less fiber than Low-N plants. No effect of N and no N*O_3 interaction was observed for concentrations of crude protein or lignin.

3.4. Plant phenology

Plant phenology was evaluated from the start of flowering until the end of plant life span (Table 1). The first evaluation was carried out at the end of O_3 exposure in OTCs, coincidentally with biomass harvest (38 DaS). At this stage, O_3 effects were not significant, but a trend was observed by which O_3 exposure accelerated *B. maxima* development: plants starting stem ear growth (stage 1) were more numerous in NFA+ treatment compared with control, in which most plants remained in the vegetative stage (stage 0) ($p < 0.1$). Thirty days after the end of O_3 exposure (60 DaS), O_3 effect on plant phenology was pronounced ($p < 0.01$). Panicles were present in 22% of the control plants (stage 3), but in NFA and NFA+ this percentage was increased to 68% (mean of both treatments). Fig. 3 shows plant phenology at 72 DaS, when *B. maxima* plants corresponding to stages 2, 3 and 4 coexisted. At this date, the O_3-induced reduction of plant life span remained significant ($p < 0.01$). Although the percentage of plants in stage 3 (green panicles) was similar in the three O_3 treatments, plants reaching stage 4 (dry panicles and mature seeds) were much more abundant in the NFA and NFA+ treatments (44% higher compared with 11% in the CFA treatment, $p < 0.01$). At 72 DaS, the number of plants that were still in stage 2 (without ears) were the most frequent in the control treatment without O_3 (52%), but limited in the NFA+ treatment (17%).

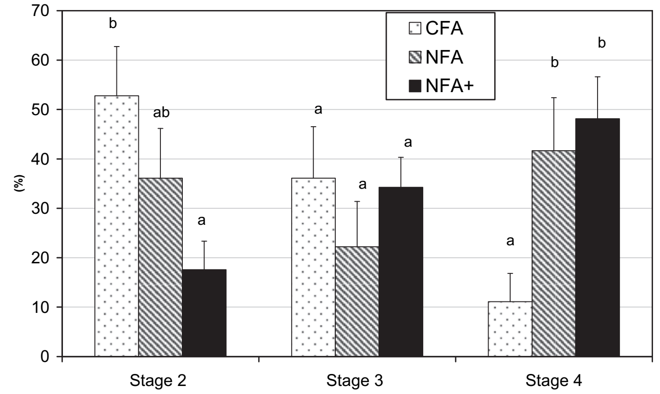

Fig. 3. Plant phenology on September 12th (44 days from the start of the blooming period). Values represent the percentage of plants in the different flowering stages (mean of all N treatments ± se): Stage 2. Start of flowering, ear stems growing although ears were not yet developed; Stage 3. Ear developing stage, plants with developing ears and greenish panicles; Stage 4. Seed ripening, plants with mature ears and dry/yellowish panicles indicating seed maturity close to anemochoric dispersion. Letters indicate significant differences among ozone treatments (Tukey test, $p < 0.05$).

Table 4
Ozone (O_3) and nitrogen supply (N) effects on nutritive quality-related parameters of *Briza maxima* at the end of the exposure period (30 days).

First assay	CP (%)	NDF (%)	ADF (%)	Lignine (%)	RFV
O_3	0.05	<0.01	< 0.001	<0.01	<0.01
Nitrogen	ns	<0.05	<0.05	ns	<0.05
*O_3 * Nitrogen*	ns	ns	ns	<0.1	ns
Low-N CFA	9.81 ± 0.49	45.20 ± 1.36	22.79 ± 0.57	0.12 ± 0.04	146.90
Low-N NFA	10.50 ± 0.31	50.36 ± 0.63	24.46 ± 0.48	0.35 ± 0.04	129.06
Low-N NFA+	9.58 ± 0.33	48.98 ± 1.18	24.20 ± 0.53	0.56 ± 0.06	133.33
Medium-N CFA	10.03 ± 0.08	43.59 ± 0.93	21.75 ± 0.50	0.19 ± 0.08	153.85
Medium-N NFA	10.44 ± 0.17	47.83 ± 0.51	24.09 ± 0.17	0.40 ± 0.05	136.43
Medium-N NFA+	9.46 ± 0.23	47.99 ± 1.28	24.13 ± 0.63	0.48 ± 0.10	136.25
High-N CFA	11.02 ± 0.83	43.02 ± 1.82	21.23 ± 0.51	0.26 ± 0.05	157.43
High-N NFA	9.88 ± 0.16	45.88 ± 0.47	23.05 ± 0.23	0.27 ±	143.88
High-N NFA+	9.06 ± 0.48	45.98 ± 0.61	22.94 ± 0.21	0.23 ± 0.04	143.77

The upper part of the table shows the results of the two-way ANOVA test that was carried out to evaluate the effects of O_3 and N, singly and in combination, on the assessed parameters. The lower part indicates the mean values and their standard errors corresponding to the different treatments. CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; RFV = relative feed value (standardized by reference to a medium-quality forage containing 53% NDF, 41% ADF and RFV of 100); CFA = Charcoal filtered air; NFA = Non-filtered air; NFA+ = Non filtered air + 40 ppb O_3; Low-N = total N supply of 5 kg ha^{-1} y^{-1}; Medium-N = total N supply of 15 kg ha^{-1} y^{-1}; High-N = total N supply of 30 kg ha^{-1} y^{-1}.

The end of the *B. maxima* life cycle was defined as the time when more than 50% of the plants were at stage 4, meaning that plant biomass was fully dried up and panicles contained mature seeds. This stage was reached earlier, 72 DaS, in the NFA+ treatment. The NFA and CFA plants needed 6 (78 DaS) and 11 (84 DaS) more days, respectively, to reach this stage. These results indicate that *B. maxima* life span was shortened by O_3 exposure. There were no nitrogen effects on *B. maxima* phenology on any of the sampling dates.

4. Discussion

B. maxima can be classified as an O_3-resistant species based on biomass response in the two experiments, since no significant O_3 effects were detected for green, total aerial, subterranean or total biomass. These results are in agreement with previous studies in which *B. maxima* showed the same relatively consistent response to O_3 when a set of 22 annual species was tested (Gimeno et al., 2004a; Bermejo et al., 2003). The greater O_3 resistance of the *Gramineae* family compared with other families, notably legumes, is also observed in experimental settings that closely approximate field conditions; i.e. involving species mixtures or managed grasslands, at least when analyses are based on species diversity or growth parameters (Wilbourn et al., 1995; Volk et al., 2005).

However, O_3 induced leaf senescence in both experiments resulted in an increased senescent/green biomass ratio. Increased senescence is a general response to numerous stresses, but it has also been described as an effect caused by O_3 (Franzaring et al., 2000; Sanz et al., 2005). Senescence is a crucial parameter for determining O_3 sensitivity of members of the *Gramineae* family, as they usually do not develop visible symptoms specific to O_3 on their leaves (Bermejo et al., 2003).

Senescence responses to O_3 were homogeneous in both assays, despite O_3 levels were higher during the first experiment compared with the second assay. But also more stressful meteorological conditions were recorded during the first assay than in the second, with higher T, VPD and solar radiation, while both assays kept adequate water availability. High T, VPD and PAR usually diminish plant gas exchange inducing stomatal closure to avoid excessive water losses and oxidative stress caused by the photoinhibition. This behaviour occurs despite sufficient soil water is available for plants, as has been described for different annual species, both *Gramineae* and *Leguminoseae*, characteristic of Dehesa communities (Alonso et al., 2007; González-Fernández et al., 2010) and other Mediterranean species (Elvira et al., 1995). In this sense, the higher T, VPD and PAR conditions during the first assay seemed to have restricted plant-atmospheric gas exchange reducing carbon availability for the photosynthetic process, resulting in the noticeably lower plant growth observed. Similarly, the lower stomatal conductance would have reduced absorbed O_3 fluxes into the plant. Therefore, the different O_3 levels and meteorological parameters prevailing in each experiment might have resulted in similar plant O_3 uptake in both assays. Studies carried out in the last decade have clearly established a closer relationship between O_3-induced effects and the actual O_3 amount absorbed by the plant (O_3 dose) compared with the O_3 levels in the atmosphere (O_3 concentration) (Pleijel et al., 2007; UN/ECE, 2008).

Ozone exposure not only accelerated plant senescence but also increased ADF, NDF and lignin concentrations and consequently reduced forrage quality (RFV) by about 10%. The NDF fraction of forage consists of partially and variable digestible cell-wall constituents (primarily cellulose, hemicellulose, lignin, silica and insoluble N) whereas the ADF fraction includes the least digestible and indigestible cell-wall constituents (NDF less hemicellulose). Thus, the greater the NDF and ADF values in forage, the lower will be its nutritive quality for ruminant herbivores when reported as RFV. Plant maturation in herbaceous species is linked to structural changes in cell-wall constituents, resulting in an increase in leaf percentage of fiber and lignin and causing a reduction in its nutritive value for herbivorous consumption (Mulholland et al., 1996). Lignin synthesis and metabolic processes related to phenolic compounds are some of the biochemical disruptions evident after O_3 exposure (Kangasjärvi et al., 1994). In agreement to these findings, the reduction observed in nutritive quality might be interpreted as an O_3-induced acceleration of the senescence process.

Few studies have analysed yield and nutritive quality responses to O_3 simultaneously, and most of them involve assays with O_3-sensitive legume species (Powell et al., 2003; Sanz et al., 2005; Muntifering et al., 2006; Lin et al., 2007). Little information is available on O_3-tolerant and/or resistant grass species. Powell et al. (2003) and Bender et al. (2006) found that O_3 exposure induced adverse effects on quality characteristics but not on yield, similarly to our findings with *B. maxima*. Also, an O_3-induced reduction in forage quality due to an increase in foliar concentrations of cell-wall components has been reported in other species (Muntifering et al., 2000; Bender et al., 2006). On the contrary, some studies did not detect any O_3 effect on forage quality when grass-clover mixtures were analysed (Pleijel et al., 1996; González-Fernández et al., 2008). Further research should be developed to define the extent and severity of O_3 effects on nutritive quality parameters of herbaceous species under field conditions where inter- and intra-species competition is also considered.

One of the most significant effects induced by O_3 exposure in *B. maxima* was the alteration of its phenology. Ozone shortened the life cycle of the grass by 18%. This result is in agreement with the response observed on senescence and nutritive quality parameters, as they indicate an overall process of accelerated plant maturation induced by the pollutant. Changes in the phenology can affect the complementarity among species in the community; i.e. the different temporal or spatial resource partitioning among species that reduces competition among neighbors facilitating species coexistence (Cleland et al., 2006; Gross et al., 2007). Annual Mediterranean pastures are characterized by a high species diversity and complex dynamics (Peco et al., 1998; Pineda et al., 2002). Annual plant growth is concentrated in a short growing season, so even small shifts in phenology can disrupt the temporal overlap of life span of species changing the community composition and/or functioning.

The observed alteration in the phenology of *B. maxima* can be considered as an O_3 carryover effect because it was expressed after the O_3 exposure under controlled conditions was terminated. Sanz et al. (2005) also reported O_3-induced carryover effects in the reproductive stage of an annual clover species resulting from early-season O_3 exposure during its vegetative stage. Carryover effects in Mediterranean-adapted species can be an important issue because they usually accumulate photosynthates in the root system at early stages to overcome stressful periods when soil moisture and nutrient availability are limited, usually during the flowering and seed production stages (Aers et al., 1991). Consequently, O_3 exposure during early plant stages can affect their late development and seed production determining next generation and species fate. These results are in contrast with the widely held concept that the most O_3 sensitive plant period in herbaceous species occurs during flower and seed development (Pleijel et al., 1998).

The characteristically low nutrient content of the soils of annual pastures on the Iberian Peninsula (Olea and San Miguel-Ayanz, 2006) might explain the strong response of *Briza* to N fertilization. *Briza* biomass was greatly enhanced by N substrate supplementation, especially aerial structures, increasing the aerial/subterranean biomass ratio. Previous experimental studies analysing the N response of other species that typically coexist with *Briza* have shown a similar strong response to N, even those belonging to the legume family (Sanz et al., 2005, 2007). N efficiency was higher in the first assay since double the dose of N was needed in the second to achieve a similar response regarding green, total aerial and total biomass. These differences might be explained by the different meteorological conditions recorded during both experiments determining plant growth. The higher T, VPD and radiation restricted plant growth during the first assay, while in the second one, the more favourable conditions for growing increased N requirements diminishing the fertilizer effect of N supplementation. As a result, the effect of N supplementation or N deposition would depend on plant physiological activity.

Briza phenology was not affected by N addition. On the other hand, the fertilizer improved the nutritive quality of forage by decreasing foliar fiber concentrations (ADF and NDF) and thus increasing RFV by ca. 9%. Because herbaceous species are a key constituent of grazing cattle diets, N supplementation of pastures may improve their production and quality, thus avoiding or reducing costs of supplemental feed and yielding greater profits. Interestingly, Sanz et al. (2005) studying an annual clover species of the same Mediterranean pastures community of *B. maxima*, found that N supplementation enhanced the nutritive quality of *Trifolium subterraneum* through increasing the crude protein concentration but without affecting fiber or lignin concentrations. The different effects of N supplementation on nutritive quality of *Gramineae* and *Leguminoseae* with different N metabolism require further research. Similar to the results observed in annual pastures, Jacobs et al. (1999, 2002) and McKenzie et al. (2003) working with grazed perennial ryegrass/white clover dairy pastures, found an improvement in nutritive quality caused by an N- induced decrease in NDF concentration and enhanced concentration of crude protein.

An increase in N availability can also modify the O_3-induced effects on grasslands. Although no interaction between O_3 exposure and N was observed in the first assay, N supplementation counteracted the adverse effect of increase senescence by the high O_3 exposure treatment in the second assay. Thus, N did not increase O_3 sensitivity of *Briza*. The little information available on the complex interaction between O_3 exposure and N substrate content on the performance of annual Mediterranean pastures contains variable results. Nitrogen protected *Trifolium subterraneum* from accelerated leaf senescence (Sanz et al., 2005), as observed in *Briza*, but enhanced detrimental effects of O_3 on its nutritive quality. Similarly, the O_3-induced decreases of plant and flower biomass and seed production of *Trifolium striatum* were partially counterbalanced by soil N enrichment but only when plants were exposed to moderate O_3 levels and not when plants were exposed to higher O_3 concentrations (Sanz et al., 2007). The variability found in the O_3 and N interaction responses, points out the need of more research involving annual communities to identify the O_3 effects on the performance of the community as a whole.

The strong O_3-induced effects observed on phenology and nutritive quality in our experiment demand a discussion about which parameters should be considered in the definition of O_3 phytotoxical thresholds for natural ecosystems. In this sense, the high AOT40 of 11,000 $\mathrm{nl\,l^{-1}\,h}$ did not reduce the yield response of *B. maxima* but the small AOT40 of 1500 $\mathrm{nl\,l^{-1}\,h}$ reduced its forage quality. Thus, the O_3 exposure required to cause negative effects on yield is well above the limits in the EU Directive (2008/50/EC) and the CLRTAP (UN/ECE, 2008) rules for the protection of vegetation. Consequently *B. maxima* should be classified as O_3-resistant. However, with regard to nutritive quality, this species should be classified as O_3-sensitive but would not be protected even by the long-term target set by the current EU legislation to protect vegetation from O_3 exposure.

5. Conclusions

Ozone exposure did not affect the biomass of *B. maxima*, but reduced its nutritive quality and shortened its life span. The reduced nutritive quality of *B. maxima* is a response of economical importance considering its role for cattle production in natural pastures. Nitrogen supplementation greatly stimulated *B. maxima* biomass production and improved its nutritive quality but did not affect its phenological development. The effects of N supplementation depended on meteorological conditions and plant physiological activity. Nitrogen counterbalanced the O_3-induced increase in senescence biomass but did not modify O_3 effects on nutritive quality.

The present study revealed ecologically and economically relevant quality parameters of *B. maxima* that are much more O_3-sensitive than yield and growth. Current yield-based critical levels do not protect *B. maxima* from O_3 negative effects. Definition of O_3 critical levels should be based on the most relevant response for each receptor; therefore, nutritive quality and phenology should be considered in new definitions of the O_3 limits for the protection of herbaceous vegetation.

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References

Aers, R., Boot, R.G.A., van der Aart, P.J.M., 1991. The relation between above- and below-ground biomass allocation. Patterns and competitive ability. Oecologia 87, 551–559.

Alonso, R., Elvira, S., Castillo, F.J., Gimeno, B.S., 2001. Interactive effects of ozone and drought stress on pigments and activities of oxidative enzymes in *Pinus halepensis*. Plant Cell and Environment 24, 905–916.

Alonso, R., Bermejo, V., Sanz, J., Valls, B., Elvira, S., Gimeno, B.S., 2007. Stomatal conductance of semi-natural Mediterranean grasslands: implications for the development of ozone critical levels. Environmental Pollution 146, 692–698.

Alonso, R., Bermejo, V., Elvira, S., Aguirre Alfaro, A., Sanz, J., Herce Garraleta, MD., González Fernández, I., Fernández Patier, R., Gimeno, B.S., 2009. La contaminación atmosférica en la sierra de Guadarrama. Riesgos potenciales para la vegetación. VI Jornadas Científicas del Parque Natural de Peñalara y del Valle de El Paular. Comunidad de Madrid, pp. 63–86.

Association of Official Analytical Chemists, 1995. Official Methods of Analysis, sixteenth ed. Association of Official Analytical Chemists, Washington, DC.

Bender, J., Muntifering, R.B., Lin, J.C., Weigel, H.J., 2006. Growth and nutritive quality of *Poa pratensis* as influenced by ozone and competition. Environmental Pollution 142, 109–115.

Bermejo, V., Gimeno, B.S., Sanz, J., de la Torre, D., Gil, J.M., 2003. Assessment of the ozone sensitivity of 22 native plant species from Mediterranean annual pastures based on visible injury. Atmospheric Environment 37, 4667–4677.

Bobbink, R., Hicks, K., Galloway, J., Spranger, T., Alkemade, R., Ashmore, M., Bustamante, M., Cinderby, S., Davidson, E., Dentener, F., Emmett, B., Erisman, J.-W., Fenn, M., Gilliam, F., Nordin, A., Pardo, L., de Vries, W., 2010. Global assessment of nitrogen deposition effects on plant terrestrial biodiversity: a synthesis. Ecological Applications 20, 30–59.

Calvo, E., Martín, C., Sanz, M.J., 2007. Ozone sensitivity differences in five tomato cultivars: visible injury and effects on biomass and fruits. Water Air and Soil Pollution 186, 167–181.

Cleland, E.E., Chiariello, N.R., Loarie, S.R., Mooney, H.A., Field, C.B., 2006. Diverse responses of phenology to global changes in a grassland ecosystem. Proceedings of the National Academy of Sciences of the United States of America 103, 13740–13744.

Derwent, R., Jenkin, M., Saunders, S., Pilling, M., Simmonds, P., Passant, N., Dollard, G., Dumitrean, P., Kent, A., 2003. Photochemical ozone formation in North West Europe and its control. Atmospheric Environment 37, 1983–1991.

Elvir, J.A., Wierman, G.B., Day, M.E., Greenwood, M.S., Fernandez, I.J., 2006. Effects of enhanced nitrogen deposition on foliar chemistry and physiological processes of forest trees at the Bear Brook Watershed in Maine. Forest Ecology and Management 221, 207–214.

Elvira, S., Alonso, R., Inclán, R., Bermejo, V., Castillo, F.J., Gimeno, B.S., 1995. Ozone effects on Aleppo pine seedlings (Pinus halepensis Mill.) grown in open-top chambers. Water, Air and Soil Pollution 85, 1387–1392.

Fenn, M.E., Jovan, S., Yuan, F., Geiser, L., Meixner, T., Gimeno, B.S., 2008. Empirical and simulated critical loads for nitrogen deposition in California mixed conifer forests. Environmental Pollution 155, 492–511.

Ferretti, M., Bussotti, F., Calatayud, V., Sanz, M.J., Schaub, M., Kräuchi, N., Petriccione, B., Sanchez-Peña, G., Ulrich, E., 2007. Ozone and forests in South-Western Europe. Environmental Pollution 145, 617–619.

Fowler, D., Cape, J.N., Coyle, M., Flechard, C., Kuylenstierna, J., Hicks, K., Derwent, D., Johnson, C., Stevenson, D., 1999. The global exposure of forest to air pollutants. Water. Air and Soil Pollution 116, 5–32.

Franzaring, J., Tonneijck, A.E.G., Koijman, A.W.N., Dueck, Th.A., 2000. Growth responses to ozone in plant species from wetlands. Environmental and Experimental Botany 44, 39–48.

Gimeno, B.S., Bermejo, V., Reinert, R., Zheng, J., Barnes, J., 1999. Adverse effects of ambient ozone on watermelon yield and physiology at a rural site in Eastern Spain. New Phytologist 144, 245–260.

Gimeno, B.S., Bermejo, V., Sanz, J., de la Torre, D., Elvira, S., 2004a. Growth response to ozone of annual species from Mediterranean pastures. Environmental Pollution 132, 297–306.

Gimeno, B.S., Bermejo, V., Sanz, J., de la Torre, D., Gil, J.M., 2004b. Assessment of the effects of ozone exposure and plant competition on the reproductive ability of three therophytic clover species from Iberian pastures. Atmospheric Environment 38, 2295–2303.

González-Fernández, I., Bass, D., Muntifering, R., Mills, G., Barnes, J., 2008. Impacts of ozone pollution on productivity and forage quality of grass/clover swards. Atmospheric Environment 42, 8755–8769.

González-Fernández, I., Bermejo, V., Alonso, R., Elvira, S., Sanz, J., Gimeno, B.S., 2010. Modelling dehesa annual pasture dynamics: application to stomatal ozone deposition. Atmospheric Environment 44, 2507–2517.

Gross, N., Suding, K.N., Lavorel, S., Roumet, C., 2007. Complementarity as a mechanism of coexistence between functional groups of grasses. Journal of Ecology 95, 1296–1305.

Gruber, N., Galloway, J.N., 2008. An earth-system perspective of the global nitrogen cycle. Nature 451, 293–296.

Jacobs, J.L., McKenzie, F.R., Ryan, M.J., Kearney, G., 1999. Effect of rate and time of nitrogen application from autumn to midwinter on perennial ryegrass–white clover dairy pastures in western Victoria. 2. Pasture nutritive value. Australian Journal of Agricultural Research 50, 1067–1072.

Jacobs, J.L., McKenzie, F.M., Kearney, G., 2002. Nitrogen fertiliser effects on perennial ryegrass nutritive characteristics, nitrate and mineral content during late autumn, mid and late winter in western Victoria. Australian Journal of Experimental Agriculture 42, 945–952.

Jones, M.E., Paine, T.D., Fenn, M.E., 2008. The effect of nitrogen additions on oak foliage and herbivore communities at sites with high and low atmospheric pollution. Environmental Pollution 151, 434–442.

Jonson, J.E., Simpson, D., Fagerli, H., Solberg, S., 2006. Can we explain the trends in European ozone levels? Atmospheric Chemistry 6, 51–66.

Kangasjärvi, J., Talvinen, J., Utriainen, M., Karjalainen, R., 1994. Plant defence systems induced by ozone. Plant Cell and Environment 17, 783–794.

Krupa, S., Muntifering, R., Chappelka, A., 2004. Effects of ozone on plant nutrive quality characteristics for ruminant animals. The Botanica 54, 1–12.

Langner, L., Bergström, R., Foltescu, V., 2005. Impact of climate change on surface ozone and deposition of sulphur and nitrogen in Europe. Atmospheric Environment 39 (6), 1129–1141.

Linn, J.G., Martin, N.P., 1989. Forage Quality Tests and Interpretation. AG-FO-02637. University of Minnesota Extension Service, Minneapolis.

Lin, J.C., Nosal, M., Muntifering, R.B., Krupa, S.V., 2007. Alfalfa nutritive quality for ruminant livestock as influenced by ambient air quality in west-central Alberta. Environmental Pollution 149, 99–103.

McKenzie, F.R., Jacobs, J.L., Kearney, G., 2003. Long term effects of multiple applications of nitrogen fertilizer on grazed dryland perennial ryegrass/white clover dairy pastures in south west Victoria. Botanical composition, nutritive characteristics, mineral content and nutrient selection. Australian Journal of Agricultural Research 54, 477–485.

Meleux, F., Solmon, F., Giorgi, F., 2007. Increase in summer European ozone amounts due to climate change. Atmospheric Environment 41, 7577–7587.

Mulholland, J.G., Nandra, K.S., Scott, G.B., Jones, A.W., Coombes, N.E., 1996. Nutritive value of subterraneum clover in a temperate environment. Australian Journal of Experimental Agriculture 36, 803–814.

Muntifering, R.B., Crosby, D.D., Powell, M.C., Chappelka, A.H., 2000. Yield and quality characteristics of bahiagrass (*Paspalum notatum*) exposed to ground-level ozone. Animal. Feed Science and Technology 84, 243–256.

Muntifering, R.B., Chappelka, A.H., Linn, J.C., LinKarnosky, D.F., Somers, G.L., 2006. Chemical composition and digestibility of *Trifolium* exposed to elevated ozone and carbon dioxide in a free-air (FACE) fumigation system. Functional Ecology 20, 269–275.

Nali, C., Francini, A., Lorenzini, G., 2006. Biological monitoring of ozone: the twenty-year Italian experience. Journal of Environmental Monitoring 8, 25–32.

Nolle, M., Ellul, R., Heinrich, G., Güsten, H., 2002. A long-term study of background ozone concentrations in the central Mediterranean-diurnal and seasonal variations on the island of Gozo. Atmospheric Environment 36, 1391–1402.

Olea, L., San Miguel-Ayanz, A., 2006. The Spanish dehesa. A traditional Mediterranean silvopastoral system linking production and nature conservation. In: Lloveras, J., González-Rodríguez, A., Vázquez-Yañez, O., Piñeiro, J., Santamaría, O., Olea, L., Poblaciones, M.J. (Eds.), Sustainable Grassland Productivity: Proceedings of the 21st General Meeting of the European Grassland Federation, pp. 1–15.

Peco, B., Espigares, T., Levassor, C., 1998. Trends and fluctuations in species abundance and richness in Mediterranean annual pastures. Applied Vegetation Science 1, 21–28.

Perez Prieto, D., Font, X., 2005. Revisión sintaxonómica a nivel de subalianza del orden Helianthemetalia Guttati en la Peninsula Ibérica e Islas Baleares. Acta Botánica Malacitana 30, 139–156.

Pineda, F.D., de Miguel, J.M., Casado, M.A., Montalvo, J., 2002. Claves para comprender la diversidad biológica y conservar la biodiversidad. In: La Diversidad Biológica de España. Prentice Hall, pp. 7–32.

Plaza, J., Pujadas, M., Artiñano, B., 1997. Formation and transport of the Madrid ozone plume. Journal of Air & Waste Management Association 47, 766–774.

Pleijel, H., Pihl Karlsson, G., Sild, E., Danielsson, H., Skärby, L., Selldén, G., 1996. Exposure of a grass-clover mixture to ozone in open-top chambers—effects on yield, quality and botanical composition. Agriculture, Ecosystems & Environment 59, 55–62.

Pleijel, H., Danielsson, H., Gelang, J., Sild, E., Sellden, G., 1998. Growth stage dependence of the grain yield response to ozone in spring wheat (*Triticum aestivum* L.). Agriculture, Ecosystems and Environment 70, 61–68.

Pleijel, H., Danielsson, H., Emberson, L., Ashmore, M.R., Mills, G., 2007. Ozone risk assessment for agricultural crops in Europe: further development of stomatal flux and flux-response relationships for European wheat and potato. Atmospheric Environment 41, 3022–3040.

Powell, M.C., Muntifering, R.B., Lin, J.C., Chappelka, A.H., 2003. Yield nutritive quality of seriacea lespedeza (*Lespedeza cuneata*) and little bluestem (*Schizachyrium scoparium*) exposed to ground-level ozone. Environmental Pollution 122, 313–322.

Ribas, A., Peñuelas, J., 2003. Biomonitoring of tropospheric ozone phytotoxicity in rural Catalonia. Atmospheric Environment 37, 63–71.

Rodà, F., Ávila, A., Rodrigo, A., 2002. Nitrogen deposition in Mediterranean forests. Environmental Pollution 118, 205–213.

Sala, O.E., Chapin III, F.J., Armesto, J.J., Berlow, E., Bloomfield, J., Dirzo, R., Huber-Sanwald, E., Huenneke, L., Jackson, R.B., Kinzig, A., Leemans, R., Lodge, D., Mooney, H., Oesterheld, M., Poff, N.L., Sykes, M.T., Walker, B.H., Walker, M., Wall, D.H., 2000. Global diversity scenarios for the year 2100. Science 287, 1770–1774.

Sanz, J., Muntifering, R.B., Gimeno, B.S., Bermejo, V., Elvira, S., 2005. Ozone and increased nitrogen supply effects on the yield and nutritive quality of *Trifolium subterraneum*. Atmospheric Environment 39, 5899–5907.

Sanz, J., Bermejo, V., Gimeno, B.S., Elvira, S., Alonso, R., 2007. Ozone sensitivity of the Mediterranean terophyte Trifolium striatum is modulated by soil nitrogen content. Atmospheric Environment 41, 8952–8962.

Sanz, M.J., Carratalá, A., Gimeno, C., Millán, M.M., 2002. Atmospheric nitrogen deposition on the east coast of Spain: relevance of dry deposition in semi-arid Mediterranean regions. Environmental Pollution 118, 259–272.

UN/ECE, 2008. Manual on methodologies and criteria for modelling and mapping critical loads and levels and air pollution effects, risks and trends. Convention on Long-range Transboundary Air Pollution. http://www.icpmapping.org/.

Van Der Wal, R., Pearce, I., Brooker, R., Scott, D., Welch, D., Woodin, S., 2003. Interplay between nitrogen deposition and grazing causes habit degradation. Ecology Letters 6, 141–146.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74, 3583–3597.

Vingarzan, R., 2004. A review of surface ozone background levels and trends. Atmospheric Environment 38, 3431–3442.

Vitousek, P.M., Aber, J.D., Howarth, R.W., Likens, G.E., Matson, P.A., Schindler, D.W., Schlesinger, W.H., Tilman, D.G., 1997. Human alteration of the global nitrogen cycle: causes and consequences. Ecology 1, 1–15.

Volk, M., Bungener, P., Contat, F., Montani, M., Fuhrer, J., 2005. Grassland yield declined by a quarter in 5 years of free-air ozone fumigation. Global Change Biology 12, 74–83.

Wilbourn, S., Davidson, A.W., Ollerenshaw, J.H., 1995. The use of an unenclosed field fumigation system to determine the effects of elevated ozone on a grass-clover mixture. New Phytologist 129, 23–32.

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Foliar senescence is the most sensitive response to ozone in *Bromus hordeaceus* and is modulated by nitrogen input

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Abstract

A study was conducted on the effect of tropospheric ozone (O_3) on soft brome (*Bromus hordeaceus*) and the modulation of its response by nitrogen (N). Two assays were conducted using open-top chambers (OTCs). Three O_3 treatments were considered: filtered air, with concentrations below background levels (charcoal-filtered air), non-filtered air (NFA) that simulates ambient O_3 concentrations, and unfiltered air to which 40 nL L^{-1} O_3 above-ambient concentrations was added (NFA+), simulating elevated values recorded in natural areas of annual pastures in the Iberian Peninsula. Three N rates were used, simulating the increase in soil N through atmospheric deposition and excreta from livestock grazing. Ozone caused an augmentation in foliar senescence, whereas green biomass was not altered; consequently, an increased senescent/green biomass ratio was produced. A stronger O_3 effect was detected in the second assay compared with the first. This was related to the estimated absorbed O_3 fluxes, which were double the value calculated in the former. Increasing N input enhanced biomass production, but its effectiveness was greater in the first assay, under less-favourable weather conditions and lower plant growth. In the first assay, the O_3 response was modulated by N availability, which mitigated the effects of O_3 to medium concentration values. In the first assay, O_3 reduced the aerial/subterranean biomass ratio, caused by a positive-trend effect on roots. Foliar concentration of lignin was increased by O_3, and *in vitro* digestibility of aerial biomass and the plant cell wall fraction tended to decrease with increasing O_3.

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Introduction

Chemical composition of the atmosphere, particularly the abundance of tropospheric ozone (O_3) and its precursors, has experienced significant changes since the beginning of the industrial revolution in the mid-eighteenth century (Hauglustaine and Brasseur, 2001). Ozone is formed primarily from photochemical reactions between its precursors, mainly nitrogen oxides (NO_x), hydrocarbons and volatile organic compounds (VOCs) generated by anthropogenic and natural activities. Its formation also requires favourable climatic conditions such as atmospheric stability with elevated solar radiation and temperature (Crutzen *et al.*, 1999). Tropospheric O_3 is considered a greenhouse gas (Mickley *et al.*, 2001) that may contribute to global warming and climate change, but the singular aspect that has led to greatly increased scientific interest during the past 30 years is its high toxicity. It is now considered the most phytotoxic air pollutant due to the large number of plant species sensitive to its high oxidative capacity (Ashmore, 2005; Hayes *et al.*, 2007). Its effects range from loss of production and quality in crop harvest (Mills *et al.*, 2007; Booker *et al.*, 2009) to reduction in forest and herbaceous vegetation growth (Davison and Barnes, 1998; Skärby *et al.*, 1998), and alterations in the structure and diversity of natural ecosystems (Davison and Barnes, 1998; Pfleeger *et al.*, 2010).

The Iberian Peninsula has experienced a progressive increase in emissions of O_3 precursors due to extensive industrial development and transport since the 1950s. This trend continues today (MARM, 2009; Fernandez-Fernandez *et al.*, 2011), despite several EU laws having been enacted in recent decades that aim

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to restrict emissions of O_3 precursors. In addition, climatic and topographical conditions of the Peninsula favour the generation of this pollutant (Castell *et al.*, 2008). All these circumstances mean that current values set by European legislation (2008/50/EC) for the protection of vegetation (MARM, 2009) are often exceeded, and they are sufficiently elevated to interfere with and disrupt production and operation of agroecosystems and natural ecosystems (Gimeno *et al.*, 1999; Bermejo *et al.*, 2003; Calvo *et al.*, 2007; Calatayud *et al.*, 2011).

In concert with increasing concentrations of O_3, another environmental problem that also has had an effect at a global scale is the increased atmospheric deposition of nitrogen (N) in ecosystems (Phoenix *et al.*, 2006). Increased use of fossil fuels in transport and industrial processes has been the main cause of emissions into the atmosphere of oxidized N compounds (NOx), while the adoption of increasingly intensive agricultural and livestock–farming systems has been responsible for the release of reduced nitrogen compounds (NH_3). Both types of compounds are causing an alteration of the N cycle and an increase in forms of N that are biologically available to plants (Stevens *et al.*, 2011). Nitrogen enrichment of anthropogenic origin is considered one of the main forces of change in Mediterranean ecosystems (Sala *et al.*, 2000). Few experimental measurements of N deposition have been performed in the Iberian Peninsula, but Rodà *et al.* (2002) estimated it as between 15 and 22 kg N ha^{-1} $year^{-1}$. This range exceeds the empirical critical load currently defined for the protection of many plant communities (Bobbink *et al.*, 2010). Nitrogen deposition can cause, among other effects, changes in the susceptibility of species to biotic or abiotic factors (Jones *et al.*, 2008), or nutrient imbalances (Elvira *et al.*, 2006). When N deposition exceeds the assimilative capacity of ecosystems, it may cause an alteration of competitive relationships between species, thereby inducing alterations in the structure and diversity of the community (Van der Wal *et al.*, 2003; Bobbink *et al.*, 2010).

The presence of elevated levels of O_3 and N enrichment are two major components of global environmental change, the combination of which can seriously compromise current functioning of ecosystems. There are extensive rural areas on the Iberian Peninsula that are subject to this combination of factors, for instance in the dehesas at the base of the Sierra de Guadarrama, north of Madrid (Plaza *et al.*, 1997; Alonso *et al.*, 2009). The dehesas, which occupy about 4 million hectares on the Iberian Peninsula, are an agrosylvopastoral system comprising a low-density arboreal stratum, under which a pasture dominated by annual species serves as food for livestock and wildlife. The pastureland of the dehesa is characterized by strong seasonal and interannual variation in biomass production and nutritional quality that is strongly correlated with precipitation variability characteristic of the Mediterranean climate (Vazquez De Aldana *et al.*, 2008). The most representative families of these pastures for both their species richness and importance for cattle grazing are *Poaceae* and *Fabaceae* (Buendía, 2000). In the last decade, several studies of species of both families have been conducted to characterize their sensitivity to O_3 and to ascertain whether increased N in the substrate modulates their response (Sanz *et al.*, 2005, 2007, 2011). These experiments have shown greater sensitivity of legumes than grasses based on parameters of biomass production, reproductive capacity and nutritional quality, and in the complexity of the interaction between O_3 and N, depending on the parameter considered and O_3 levels (Bermejo *et al.*, 2003; Gimeno *et al.*, 2004a,b). Another noteworthy result is the sensitivity to O_3 of parameters such as nutritional quality or phenology in species that show no visible foliar damage or effects on biomass production (Sanz *et al.*, 2011). The results indicate the potential sensitivity of these Mediterranean grasslands to the O_3 and N factors considered, and this may cause changes in the competitive ability of species by altering their structure and composition or interfering with natural nutrient cycles. The loss of nutritional quality can have important implications for feeding cattle, or for wild herbivores (Krupa *et al.*, 2004; Gilliland *et al.*, 2012), although this is not currently considered as a parameter for defining critical O_3 values for protecting semi-natural ecosystems (CLRTAP, 2010).

This study is framed within an extensive line of work devoted to analysing the sensitivity of Mediterranean grasslands to global change. It aims to determine which species/communities are most sensitive, how generalizable are the responses found, and which parameters must be considered for characterization of this sensitivity and which should be used to define the value limits of pollutants to ensure protection of plant communities (levels and critical loads of O_3 and N). This article specifically presents the study conducted using *Bromus hordeaceous*, a characteristic annual grass of dehesa pastures. It starts with the assumption that *B. hordeaceous* would behave similarly to other previously analysed annual grasses and, therefore, should be relatively resistant to O_3 considering biomass production parameters, although greater sensitivity to O_3 of the parameters related to nutritional quality and a possible modulation of response by N might be expected.

Materials and methods

Experimental design

The experiment was conducted in an experimental facility of OTCs, a system designed specifically to analyse effects of air pollutants on vegetation. The experimental field was located in a rural area distant from sources of pollution in the north-east of the Iberian Peninsula on the Ebro delta (latitude 40°41′N; longitude 0°47′E; 2 m elevation; Tarragona). An automatic and sequential system allowed continuous monitoring of concentrations of O_3, SO_2, NO and NO_2 inside each of the chambers. The system allowed 10 min for sampling the pollution climate within each OTC before changing to the next. Nine NCLAN-type OTCs were used in the assays, 3 m height × 3 m diameter. For a detailed description of the facility, see Gimeno *et al.* (1999). The Fangar weather station (40°47′N, 0°46′E), belonging to the Catalan Meteorological Service network, was used to obtain the meteorological parameters considered for the description of the experiment (temperature, relative humidity, solar radiation). Two successive assays were conducted using the same experimental design and protocol, although under different environmental conditions resulting from the different implementation dates of the assays (Table 1).

Table 1 Timetable of events for the *Bromus* assays carried out in 2002 (DS indicates days from the start of the experiment).

Event	Date	DS
First assay		
Sowing	27 May	
Emergence	29 May	
N fertilization	29 June	
Transplantation	1 July	
Start of ozone exposure in OTCs	2 July	0
N fertilization	15 July	13
N fertilization	29 July	27
End of OTC exposure	7 August	36
Harvest	7 August	36
Second Assay		
Sowing	19 September	
Transplantation	30 September	
Start of ozone exposure in OTCs	1 October	0
N fertilization	2 October	1
N fertilization	21 October	20
N fertilization	8 November	39
End of OTC exposure	12 November	43
Harvest	14 November	45

OTCs, open-top chambers.

Plant material

Seeds originated from a collection campaign conducted during May–June 2001 in the Moncalvillo Dehesa, located in San Agustín de Guadalix, in the central area of the Iberian Peninsula (40°40′N, 0°47′E). Seeds were stored free of impurities under suitable environmental storage conditions until time of use. The seeds were sown in a substrate mixture of 50% vermiculite and 50% peat. Approximately 30 days later, the seedlings were transplanted individually into 2·5-L volume pots with a mixture of 50% peat, 20% vermiculite and 30% perlite, to which $CaCO_3$ was added to adjust pH. All nutrients necessary for balanced development of the plants were provided through a nutrient solution prepared with a water-soluble, low-nitrogen fertilizer (Peters Professional®, Scotts, OH, USA; NPK: 4:25:35 plus microelements). Throughout the experiment, a drip irrigation system supplied water to the substrate. A nursery of 81 pots per assay was prepared to achieve an adequate number of samples per O_3 and N treatment.

Ozone and nitrogen treatments

Nitrogen was applied in three doses at approximately 14-day intervals, based on various solutions prepared using NH_4NO_3 (35% N; Table 1). The volume of the applied solution per pot was the same, independent of the N dose. Three treatments with total N input were considered: 7·5 (N-low), 22·5 (N-medium) and 45 (N-high) kg N ha^{-1}. These N-integrated doses were selected to reflect the natural variability that occurs in the annual N inputs to the dehesa soils of the Iberian Peninsula, considering both the N entering via excreta (Olea and San Miguel-Ayanz, 2006) and expected atmospheric N deposition (Rodà *et al.*, 2002).

Ozone treatments were the following: charcoal-filtered air (CFA), non-filtered air (NFA) that simulates ambient O_3 concentrations (NFA) and NFA to which 40 nL L^{-1} O_3 was added from 07:00 until 17:00 (GMT) 5 days a week (NFA+), which simulates the elevated O_3 values that occur in the natural growing areas of this species. Three replicates per O_3 treatment (three OTCs per treatment) and three plants per N treatment within each OTC were used; therefore, there were nine plants for each combination of O_3 and N.

Biomass parameters

Aerial biomass was obtained by cutting the plant flush with the substrate after 37 and 45 days for the first and second assays, respectively, inside the chambers and exposed to the different O_3 treatments. Senescent biomass, including both senesced leaves and senesced part of the leaves, was separated from green biomass to calculate the ratio between the two. Roots were collected by careful continuous washing in the substrate water. Aerial and subterranean biomass was dried at 60°C to constant weight.

Nutritional quality parameters

Nutritional quality parameters were analysed in the first assay based on samples collected for analysis of green biomass. Green biomass of three pots per chamber and N treatment were pooled for quality analysis. Crude protein (CP) concentration (% CP = % N × 6·25) was determined by the Kjeldahl method (Association of Official Analytical Chemists, 1995). Concentrations of neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin were determined sequentially following the procedures of Van Soest *et al.* (1991). Relative feed value (RFV) was calculated from ADF and NDF concentrations using prediction equations developed for cool-season-adapted C_3 grasses and legumes (Linn and Martin, 1989). *In vitro* dry-matter digestibility (IVDMD) was determined according to the Van Soest *et al.* (1991) modification of the two-stage Tilley and Terry (1963) procedure, in which neutral detergent extraction of 48-hour fermentation residues was substituted for acid-pepsin digestion. *In vitro* NDF digestibility (IVNDFD) was calculated as $[(\text{NDF}_i - \text{NDF}_r) \div \text{NDF}_i] \times 100\%$, where NDF_i represents the initial mass of NDF in the sample and NDF_r represents the final mass of NDF residue following extraction with neutral detergent solution. Ruminal fluid used for *in vitro* batch culture fermentation assays was obtained from a fistulated Holstein cow fed a mixed forage–grain concentrate diet.

Statistical analysis

The effect of fertilization with N and O_3 concentrations on the parameters considered was analysed by a two-way analysis of variance for each parameter. The Tukey's test was used to analyse differences among means when ANOVA indicated significant differences ($P < 0·05$). Prior to the ANOVA, homogeneity of variances was checked by means of the Levene's test and normality of the variables and their residues were analysed based on the corresponding graphs. The presence of influential points (leverage) was also analysed. Logarithmic transformation was performed for variables that did not meet some of these requirements.

Calculation of phytotoxic ozone dose

Ozone-absorbed fluxes inside the plant or phytotoxic O_3 dose (POD) were calculated following the criteria developed under the Convention on Long-range Transboundary Air Pollution (CLRTAP, 2010). Stomatal conductance was modelled using a multiplicative approach (Jarvis, 1976) parameterized for *B. hordeaceus* by Alonso *et al.* (2007). In the stomatal conductance model, f_{phen} and f_{SWP} were set to 1, and $g_{\max}$ was transformed to mmol O_3 m^{-2} s^{-1} using the coefficient of diffusivity factors of H_2O and O_3 in air, 0·663 (Massman, 1998). POD was accumulated from the start of the O_3 fumigation treatments until the end of the experiment. No flux thresholds were considered for the calculation of the total POD.

Results

Meteorological conditions and ozone levels

The mean values of meteorological parameters for the two assays are summarized in Table 2. In the first assay, the values of atmospheric vapour pressure deficit (VPD) were greater due to temperature, which maintained a mean value about 6°C above those recorded in the second assay for most of the day, whereas relative humidity (RH) conditions were similar in both (a mean value between 70 and 90% throughout the day). Another difference between assays was the total solar radiation (SR, Wm^{-2}), as during the first assay, more hours of more intense sunlight were accumulated. These differences may be key factors in attempting to explain some

Table 2 Mean, maximum and minimum meteorological values and AOT40 index for the *Bromus* assays.

	Mean	Max.	Min.
First assay			
	24·5	32·0	17·7
Temperature (°C)			
RH (%)	83	100	30
SR (Wm^{-2})	257	948	0
VPD (Kpa)	0·55	3·04	0
AOT40 (nL L^{-1} h) CFA	39		
AOT40 (nL L^{-1} h) NFA	1198		
AOT40 (nL L^{-1} h) NFA+	10 891		
Second assay			
Temperature (ºC)	18·4	25·6	11·9
RH (%)	84	100	51
SR (Wm^{-2})	142	750	0
VPD (Kpa)	0·36	1·52	0
AOT40 (nL L^{-1} h) CFA	0		
AOT40 (nL L^{-1} h) NFA	221		
AOT40 (nL L^{-1} h) NFA+	6032		

RH, air relative humidity; SR, total solar radiation; VPD, atmospheric water vapour pressure deficit; CFA, charcoal-filtered air; NFA, non-filtered air; NFA+, non-filtered air + 40 nL L^{-1} O_3.

of the differences observed between assays in response to O_3 and N factors, because both VPD and SR are variables that strongly affect stomatal opening and therefore absorption of O_3 and CO_2 into the plant.

Although the exposure period to O_3 of the second assay was 7 days longer than in the first one, AOT40 accumulative exposure index values of O_3 were significantly lower in this period (Table 2). Differences are due to different ambient O_3 levels between assays. In the first assay, the AOT40 value registered in the NFA treatment, which reproduces ambient levels of the pollutant, was six times higher than in the second assay, whereas the treatment with higher levels of O_3 (NFA+) was double. Values for the filtered control treatment (CFA) were consistently below 10 nL L^{-1} in both exposure periods, indicating proper operation of the filtration system. Ozone levels reproduced in the different treatments are within the seasonal and inter-annual variability recorded for this pollutant in the areas of distribution of annual grasslands in the central area of the Iberian Peninsula. Nitrogen dioxide and SO_2 were always below very low levels and close to the detection limit of the monitors (data not shown). Total N deposition in the area, according to the EMEP model (EMEP MSC-W chemical transport model, 2012; http://www.emep.int/) was around 6·75 kg N ha^{-1} $year^{-1}$ for the year of the experiment.

Biomass production

First assay

Mean values and results of statistical analyses of the first experiment for the various parameters of biomass analysed, by O_3 and N treatments, are presented in Table 3. Exposure to O_3 did not affect production of green biomass, but significantly increased the production of senescent biomass ($P < 0{\cdot}005$), which increased by 7 and 34% for NFA and NFA+ treatments, respectively, compared with CFA (mean values across N treatments), but differences with NFA were not significant. These results caused an imbalance in the senescent biomass/green biomass ratio ($P < 0{\cdot}005$), which increased 15 and 50% in plants grown in NFA and NFA+ respectively. Total aerial biomass (sum of green and senescent biomass) was not, however, significantly affected by the pollutant. Although the effect of O_3 on subterranean biomass was not significant, a trend was observed ($P = 0{\cdot}07$) towards an increase in the NFA+ treatment, which caused a significant reduction ($P < 0{\cdot}05$) of 14% (mean across N treatments) in the aerial/subterranean biomass ratio in plants exposed to the highest levels of pollutant ($P < 0{\cdot}05$). The O_3 increased the fresh green/dry weight ratio in NFA and NFA+ compared with the CFA control by 8% (mean value across N treatments), indicating greater water content in green aerial biomass.

Nitrogen stimulated growth progressively in all analysed biomass parameters commensurate with dose increase, except for senescent biomass that only responded to the highest dose of N. Total biomass (sum of aerial and root biomass) was increased by 30% for N-medium and 72% for N-high, as compared to that of N-low (mean across O_3 treatments). The fertilizer elicited greater growth response by the aerial part than the subterranean part. Mean values for total aerial biomass were 34 and 90% greater for N-medium and N-high, respectively, than N-low (mean across O_3 treatments); however, root biomass increased by 26 and 57%, respectively, in N-medium and N-high treatments compared with N-low. These imbalanced effects were reflected in the aerial biomass/root biomass ratio, which increased by 19% in N-high ($P < 0{\cdot}05$).

The senescent/green ratio decreased by 29 and 38% for N-medium and N-high, respectively, compared with the lowest dose of N ($P < 0{\cdot}001$; mean values across O_3 treatments), thereby producing the opposite effect to that of O_3, which caused an increase in this ratio (33% in NFA+). The only parameter in which a significant interaction ($P < 0{\cdot}05$) was observed among the O_3 and N factors was the senescent/green biomass ratio, in that N attenuated the increase in the ratio induced by high O_3 exposure levels (NFA+; Figure 1).

Second assay

In the second assay, biomass production of *B. hordeaceous* was nearly double that collected in the first assay (Table 4). In this case, the negative effect of O_3 significantly affected fewer parameters related to biomass production compared with the first assay. Neither subterranean biomass nor the aerial/subterranean biomass–parameters ratio responded to increasing levels of the pollutant. Nevertheless, damage severity (percentage of effect compared with control) of affected parameters was somewhat greater than that observed in the first growth cycle. As in the first assay, O_3 caused an increase in senescent biomass, which achieved 54% (mean across N treatments) in NFA+ compared with both NFA and CFA, without affecting production of green aerial biomass. Consequently, there was a 58% increase ($P < 0{\cdot}001$) in senescent/green biomass ratio in the treatment with the greatest concentration of pollutant (NFA+) compared with the other two. No effect from O_3 was found in this assay in relation to the hydric status of biomass.

Nitrogen fertilization also caused a clear response in *B. hordeaceous* and increased the values of all biomass

Table 3 Growth-related parameters (means per plant ± s.e.) corresponding to the exposure of *Bromus hordeaceus* to the different O_3 and N treatments on the first assay.

First assay	Green biomass (g dw)	Senescent biomass (g dw)	Senescent/green biomass ratio	Total aerial biomass (g dw)	Subterranean biomass (g dw)	Total biomass (g dw)	Aerial/ subterranean ratio	Fresh aerial biomass (g fw)	Fresh/dry biomass ratio
O_3	ns	<0·005	<0·005	ns	0·07	ns	<0·05	ns	<0·005
Nitrogen	<0·0001	<0·05	<0·0005	<0·0001	<0·0001	<0·0001	<0·05	<0·0001	ns
O_3* Nitrogen	ns	ns	<0·05	ns	ns	ns	ns	ns	ns
N-low CFA	0·95 ± 0·06	0·17 ± 0·02	0·19 ± 0·03^{a}	1·12 ± 0·06	1·26 ± 0·10	2·38 ± 0·15	0·92 ± 0·06	4·52 ± 0·22	4·47 ± 0·08
N-low NFA	1·06 ± 0·08	0·17 ± 0·01	0·18 ± 0·03^{a}	1·24 ± 0·07	1·41 ± 0·05	2·64 ± 0·11	0·87 ± 0·03	6·37 ± 0·59	5·23 ± 0·19
N-low NFA+	0·85 ± 0·09	0·25 ± 0·02	0·31 ± 0·03^{b}	1·09 ± 0·10	1·54 ± 0·10	2·64 ± 0·19	0·70 ± 0·05	4·92 ± 0·39	4·99 ± 0·11
N-medium CFA	1·34 ± 0·10	0·17 ± 0·01	0·14 ± 0·01^{a}	1·52 ± 0·11	1·66 ± 0·09	3·18 ± 0·19	0·91 ± 0·04	6·93 ± 0·53	4·62 ± 0·10
N-medium NFA	1·29 ± 0·11	0·17 ± 0·02	0·14 ± 0·01^{a}	1·46 ± 0·12	1·71 ± 0·11	3·17 ± 0·22	0·85 ± 0·04	6·98 ± 0·71	4·94 ± 0·17
N-medium NFA+	1·39 ± 0·17	0·25 ± 0·02	0·21 ± 0·03^{ab}	1·64 ± 0·18	1·94 ± 0·15	3·58 ± 0·32	0·83 ± 0·05	8·18 ± 0·80	4·93 ± 0·12
N-high CFA	2·12 ± 0·25	0·21 ± 0·02	0·11 ± 0·01^{a}	2·33 ± 0·26	2·19 ± 0·14	4·52 ± 0·37	1·05 ± 0·08	11·63 ± 0·91	4·65 ± 0·05
N-high NFA	1·55 ± 0·12	0·25 ± 0·04	0·17 ± 0·03^{a}	1·80 ± 0·11	2·00 ± 0·15	3·80 ± 0·24	0·93 ± 0·07	8·43 ± 0·61	4·72 ± 0·12
N-high NFA+	2·19 ± 0·32	0·26 ± 0·02	0·13 ± 0·01^{a}	2·45 ± 0·33	2·43 ± 0·25	4·88 ± 0·57	1·00 ± 0·05	13·01 ± 2·07	4·87 ± 0·13

CFA, charcoal-filtered air; NFA, non-filtered air; NFA+, non-filtered air + 40 nL L^{-1} O_3; Low-, Medium- and High-N represent N supply at 7.5, 22.5 and 45 kg ha^{-1} respectively. Different letters indicate significant differences among means in each nitrogen treatment when interactive effects were significant.The P values corresponding to the effect of each factor (O_3 and N) are presented in the first part of the table and the values (mean ± s.e.) are presented below.

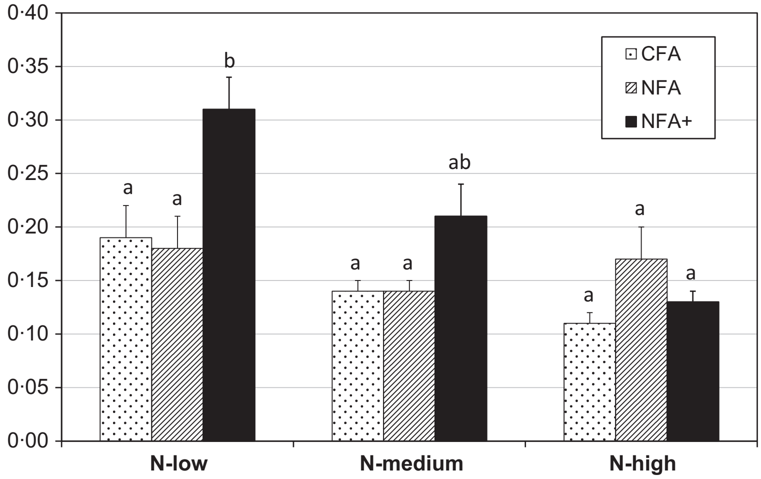

Figure 1 Mean values of senescent/green biomass ratio for the different ozone and nitrogen treatments (mean ± s.e.) for the first assay. CFA, charcoal-filtered air; NFA, non-filtered air; NFA+, non-filtered air + 40 nL L^{-1} O_3; N-low, total N supply of 7·5 kg ha^{-1}; N-medium, total N supply of 22·5 kg ha^{-1}; N-high, total N supply of 45 kg ha^{-1}. Different letters indicated significant differences among means for the NxO_3 interaction.

parameters analysed, although differences found between doses of N were less than those observed in the first assay; in fact, no significant differences were observed between N-low and N-medium. The N-high treatment similarly increased production of green and senescent biomass by 30% (mean across O_3 treatments) compared with the N-medium/N-low mean. Hence, no alteration in the senescent/green biomass ratio was observed.

Although N more strongly stimulated the aerial part of the plant (31% greater in total biomass of N-high compared with N-low) with respect to the subterranean portion (24% increase), variability among samples does not permit detection of a significant effect on aerial/subterranean biomass ratio as clearly detected in the first cycle. Plants exposed to the medium and high doses of N increased the hydration of their green biomass relative to the N-low plants, as the fresh green/dry weight ratio displayed a significant average increase of 4% for these treatments compared with N-low ($P < 0·05$). No significant interaction between O_3 and fertilizer was found in this second assay.

Nutritional quality

The mean values within O_3 and N treatments for nutritional quality parameters analysed in the first assay are presented in Table 5. Neither O_3 nor N affected foliar concentrations of CP, ADF or NDF. Concentrations of lignin in plants grown in NFA were 50% lower (mean across N treatments) than NFA+, but there was no difference between the control (CFA) and NFA+ treatments. The predicted RFV was not altered by either O_3 or N, but IVDMD and IV-NDFD both tended to decrease with increasing O_3.

Phytotoxic ozone dose

POD values for both assays are presented in Table 6. During the first assay, POD values reached 7·8 and 13·0 mmol O_3 m^{-2} s^{-1} for NFA and NFA+ respectively. Higher values were found during the second assay. In this case, POD was 14·2 and 23·2 mmol O_3 m^{-2} s^{-1} for NFA and NFA+ respectively. The O_3 absorbed fluxes during the second assay almost doubled the dose absorbed during the first assay, with an average flux increment of 80% for NFA and NFA+ in the second assay compared with the first one.

Discussion

The response of *B. hordeaceous* to O_3 followed a similar pattern in the two assays. In neither case did the pollutant alter the green aerial biomass or total aerial biomass of the species. This response concurs with results from other studies with *Poaceae*, whether annual species belonging to the same community and ecological characteristics, such as *Briza maxima* (Sanz *et al.*, 2011), or perennial species, such as *Poa pratensis*, *Anthoxantum odoratum*, *Lolium perenne* or *Dactylis glomerata* (Bender *et al.*, 2006; Gonzalez-Fernandez *et al.*, 2008; Dawnay and Mills, 2009; Wyness *et al.*, 2011). These results reinforce the generalization of lower sensitivity to O_3 in grasses than in legumes and forbs (Wilbourn *et al.*, 1995; Gimeno *et al.*, 2004a,b; Hayes *et al.*, 2010). However, increased O_3 did cause elevated premature leaf senescence, the

Table 4 Growth-related parameters (means per plant ± s.e.) corresponding to the exposure of *Bromus hordeaceus* to the different O_3 and N treatments on the second assay. (Abbreviations: refer to footnote of Table 3).

Second assay	Green biomass (g dw)	Senescent biomass (g dw)	Senescent/green biomass ratio	Total aerial biomass (g dw)	Subterranean biomass (g dw)	Total biomass (g dw)	Aerial/ subterranean ratio	Fresh aerial biomass (g fw)	Fresh/dry biomass ratio
O_3	ns	<0·0001	<0·001	ns	ns	ns	ns	ns	ns
Nitrogen	<0·0001	<0·05	ns	<0·0001	<0·0001	<0·0001	ns	<0·0001	<0·05
$O_3 \times$ Nitrogen	ns	ns	ns	ns	ns	ns	ns	ns	ns
N-low CFA	2·89 ± 0·12	0·23 ± 0·03	0·08 ± 0·01	3·12 ± 0·12	2·82 ± 0·16	5·94 ± 0·19	1·14 ± 0·08	14·97 ± 0·73	5·17 ± 0·10
N-low NFA	2·76 ± 0·12	0·20 ± 0·01	0·07 ± 0·00	2·97 ± 0·13	2·76 ± 0·17	5·73 ± 0·29	1·09 ± 0·04	14·06 ± 0·57	5·10 ± 0·07
N-low NFA+	3·00 ± 0·16	0·36 ± 0·07	0·13 ± 0·03	3·36 ± 0·14	2·99 ± 0·11	6·36 ± 0·17	1·14 ± 0·07	15·88 ± 1·18	5·24 ± 0·12
N-medium CFA	3·12 ± 0·16	0·23 ± 0·02	0·07 ± 0·01	3·36 ± 0·15	2·94 ± 0·18	6·30 ± 0·21	1·18 ± 0·09	17·30 ± 0·73	5·51 ± 0·14
N-medium NFA	3·09 ± 0·12	0·22 ± 0·02	0·07 ± 0·00	3·30 ± 0·12	3·28 ± 0·16	6·59 ± 0·23	1·02 ± 0·05	16·51 ± 0·58	5·37 ± 0·12
N-medium NFA+	2·96 ± 0·12	0·35 ± 0·03	0·12 ± 0·01	3·31 ± 0·11	3·00 ± 0·12	6·31 ± 0·15	1·12 ± 0·05	16·01 ± 0·71	5·30 ± 0·05
N-high CFA	3·88 ± 0·06	0·29 ± 0·03	0·07 ± 0·00	4·17 ± 0·06	3·57 ± 0·17	7·74 ± 0·21	1·19 ± 0·05	21·18 ± 0·50	5·45 ± 0·08
N-high NFA	3·79 ± 0·13	0·31 ± 0·01	0·08 ± 0·00	4·10 ± 0·12	3·73 ± 0·18	7·83 ± 0·21	1·12 ± 0·06	20·03 ± 0·70	5·29 ± 0·11
N-high NFA+	3·65 ± 0·14	0·43 ± 0·02	0·12 ± 0·01	4·07 ± 0·13	3·31 ± 0·13	7·39 ± 0·23	1·24 ± 0·05	19·62 ± 0·95	5·37 ± 0·09

CFA, charcoal-filtered air; NFA, non-filtered air.

mean increase in which across the two assays was 44% in the highest O_3 treatment. The increase in O_3-induced senescent biomass is a frequently observed response in many herbaceous species pertaining to different plant families (Franzaring *et al.*, 2000; Bermejo *et al.*, 2003; Sanz *et al.*, 2007). It has been considered a particularly important response parameter for defining sensitivity of grasses to O_3, which often do not develop other more specific foliar symptoms (Bermejo *et al.*, 2003; Hayes *et al.*, 2010). In the present study, O_3-enhanced leaf senescence caused an imbalance between the senescent biomass/green biomass ratio, increasing the mean value over both assays by 54% in plants grown in NFA+. Interestingly, a significant interaction between O_3 and N was found in this parameter; hence, in the first assay, the fertilizer compensated for the increased senescent/green ratio induced by exposure to elevated O_3 (NFA+). A similar response has been found in other herbaceous annuals belonging to the legume and grass annuals (Sanz *et al.*, 2005, 2011), perennial grasses (Jones *et al.*, 2010) and even trees (Bielenberg *et al.*, 2001), linking the acceleration of foliar senescence induced by O_3 to the low availability of soil N. The importance of this relatively generalized effect at the ecosystem level of organization is yet to be defined, but its impact on soil metabolic processes and nutrient cycling of the system can be significant.

Modulation of the response to O_3 by N, favouring plant development in polluted environments, has also been found in other highly important parameters for annual species such as floral biomass and seed production (Sanz *et al.*, 2007). The mechanisms by which N counterbalances the O_3 effects are still under study. The synthesis of antioxidant compounds at the cellular scale to prevent the oxidative processes induced by O_3 inside the plant tissues, or the processes related to the repair and replacement of structures affected by the pollutant, demand a metabolic cost which might be supported by the soil N availability (Andersen, 2003). In other cases, however, the interaction between O_3 and N was negative for herbaceous species, increasing the O_3-induced effects on quality parameters (Sanz *et al.*, 2005) or in the root system (Wyness *et al.*, 2011). Further studies are needed to investigate the combined effects of O_3 and N, due to the complexity of their effects.

Although the response of *B. hordeaceous* to O_3 showed a similar pattern in the two experiments, the most pronounced effects on the senescence-related parameters were found in the second experiment (i.e. senescent biomass in the first assay was 34% in the NFA+ treatment, but increased to 54% in the second). In the second assay, O_3 levels were lower and plants grew nearly double in size compared with the first assay. Increased O_3 effects in this assay could be explained by differences in environmental conditions

Table 5 Ozone (O_3) and nitrogen-supply (N) effects on nutritive quality-related parameters of *Bromus hordeaceus* at the end of the exposure period (30 days) for the first assay.

First assay	CP (%)	NDF (%)	ADF (%)	Lignin (%)	RFV	IVDMD (%)	IVNDFD (%)
O_3	ns	ns	ns	<0·05	ns	0·08	0·07
Nitrogen	ns	ns	ns	ns	ns	ns	ns
O_3 * Nitrogen	ns	ns	ns	ns	ns	ns	ns
N-low CFA	11·86 ± 1·24	51·23 ± 1·21	26·03 ± 0·69	0·84 ± 0·05	124·91 ± 8·51	85·88 ± 0·76	72·42 ± 1·38
N-low NFA	11·06 ± 1·20	51·66 ± 0·67	26·46 ± 0·34	0·48 ± 0·07	123·12 ± 2·55	85·62 ± 0·58	72·13 ± 1·14
N-low NFA+	9·25 ± 0·86	51·49 ± 0·79	26·71 ± 0·63	0·59 ± 0·04	123·14 ± 5·77	84·80 ± 0·00	69·87 ± 0·00
N-medium CFA	10·14 ± 0·75	50·85 ± 0·54	26·32 ± 0·52	0·82 ± 0·03	125·21 ± 4·20	87·02 ± 0·25	74·48 ± 0·31
N-medium NFA	11·16 ± 0·80	51·49 ± 0·74	25·77 ± 0·64	0·42 ± 0·03	124·49 ± 5·74	86·07 ± 0·40	72·97 ± 0·61
N-medium NFA+	10·03 ± 0·29	52·62 ± 0·43	26·93 ± 0·20	0·93 ± 0·08	120·10 ± 1·78	84·26 ± 0·56	70·35 ± 1·20
N-high CFA	9·89 ± 0·61	50·82 ± 0·57	26·54 ± 0·56	0·62 ± 0·04	125·00 ± 4·58	87·71 ± 0·46	75·76 ± 1·15
N-high NFA	9·54 ± 0·33	50·51 ± 0·84	25·56 ± 0·54	0·48 ± 0·08	127·20 ± 6·18	86·40 ± 0·54	73·08 ± 0·89
N-high NFA+	11·15 ± 0·48	50·51 ± 1·00	25·90 ± 0·66	1·19 ± 0·04	126·75 ± 7·25	86·05 ± 1·51	70·97 ± 2·50

The upper part of the table shows the results of the two-way ANOVA test that was carried out to evaluate the effects of O_3 and N, singly and in combination, on the assessed parameters. The lower part indicates the mean values and their standard errors corresponding to the different treatments.CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; RFV, relative feed value (standardized by reference to a medium-quality forage containing 53% NDF, 41% ADF and RFV of 100); IVDMD, *in vitro* dry-matter digestibility; IVNDFD, *in vitro* NDF digestibility; CFA, charcoal-filtered air; NFA, non-filtered air; NFA+ is non-filtered air + 40 nL L^{-1} O_3; N-low is total N supply of 5 kg ha^{-1}; N-Medium is total N supply of 15 kg ha^{-1}; N-high is total N supply of 30 kg ha^{-1}.

Table 6 Phytotoxic ozone dose (POD) accumulated from the start of the O_3 exposure till the end of the harvest for the different O_3 treatments. (CFA, charcoal-filtered air; NFA, non-filtered air; NFA+, non-filtered air + 40 nL L^{-1} O_3.) No thresholds were considered for the calculation of the total POD (POD0).

Bromus	1st assay			2nd assay		
hordeaceus	CFA	NFA	NFA+	CFA	NFA	NFA+
POD0 (mmol O_3 m^{-2} s^{-1})	1·7	7·8	13·0	2·5	14·2	23·2

under which the two experiments were conducted. In the first assay, weather conditions were more stressful for plant growth: higher temperature, solar radiation and VPD and lower relative humidity values would be more limiting to stomatal opening, thereby reducing gas exchange to optimize plant water use and reduce oxidative stress caused by photoinhibition. This behaviour has been described in herbaceous annuals that cohabit with *B. hordeaceous* in dehesa grasslands (Alonso *et al.*, 2007; González-Fernández *et al.*, 2010). This limitation of gas exchange would produce a reduction in the availability of carbon for the photosynthetic process and, consequently, reduced plant growth. Alternatively, the restriction of gas flow would have reduced O_3 uptake into the interior of cells, thereby leading to diminished negative effect of O_3. This hypothesis is supported by the estimated POD values for the two assays, showing 80% higher POD associated with more severe O_3 effects in the second assay. Studies conducted in recent decades indicate that effects caused by O_3 on vegetation are more related to the actual flux of the pollutant absorbed through stomata and not on atmospheric pollutant concentrations (Pleijel *et al.*, 2007; CLRTAP, 2010). The results of this study support this conclusion.

Ozone exposure frequently modifies the distribution of assimilated carbon, inducing an imbalance in the aerial/subterranean biomass ratio of the plant. However, the O_3 response of this ratio does not follow a common pattern among the different herbaceous species. Some species, such as *Briza maxima*, did not show O_3 effects on root biomass (Sanz *et al.*, 2011), whereas the perennial *D. glomerata* decreased root biomass, but without significant impact on aerial/subterranean biomass ratio (Wyness *et al.*, 2011). Although in a previous screening study with annuals, no O_3 effects were found in *Bromus* regarding this ratio (Gimeno *et al.*, 2004a), in the first assay of the present study O_3 induced a 14% reduction. This imbalance is associated with increased root biomass in plants subjected to high concentrations of O_3 without affecting significantly the total aerial biomass. This response can give *B. hordeaceous* some competitive advantage taking into account the opposite response found for some legumes belonging to the same natural community: the annual clover *Trifolium subterraneum* increased the aerial/subterranean ratio up to 39% caused by the more pronounced effect of O_3 on roots (Sanz *et al.*, 2007; Vollsnes *et al.*, 2010). Moreover, exposure to O_3 enhanced the foliar hydric content of *B. hordeaceous,* another contrasted response compared with other more O_3-sensitive species from the same habitat, which showed no response (Sanz *et al.*, 2011), or responded in the opposite direction (Sanz *et al.*, 2005). If future studies confirm the pattern found for *B. hordeaceous*, it would be of interest to consider the potential competitive advantage that this species could have in environments subjected to chronic O_3 contamination vs. legumes with which it competes, especially considering that it is a plant community characterized by a high diversity of species in an environment of limited water and resources (Olea and San Miguel-Ayanz, 2006).

Another aspect of great interest and importance is the analysis of the O_3 and N effects in relation to the nutritive quality of *B. hordeaceous* for herbivores. The effect of O_3 on the diet of herbivores via intake of grass exposed to elevated O_3 concentrations is a recently emerging line of research, results of which link the effects of O_3 on vegetation with its impact on the food chain (Krupa *et al.*, 2004; Gilliland *et al.*, 2012). In temperate pastures, *B. hordeaceous* is of relatively low nutritive value and has limited acceptability to grazing herbivores (Peeters, 2004). In the context of Mediterranean grasslands, however, it can be a useful forage species and it matures later than many other annual grasses. Furthermore, as its seeds do not readily shatter, cattle will graze it well into summer and derive additional nutritional benefit even after seeds have matured. Exposure to O_3 did not affect the chemical composition or nutritional quality of the leaves as indicated by the RFV index, which is calculated by reference to a digestible DM intake of a mature forage containing 53% NDF and 41% ADF equivalent to an RFV of 100 (Linn and Martin, 1989). Among all the chemical compositional parameters analysed that are related to nutritional quality (i.e. CP, ADF, NDF and lignin), only lignin was responsive to O_3. The negative relationship that exists between lignin concentration and nutritive quality is not reflected explicitly in calculation of RFV, but it is implied from the negative relationship that exists between lignin concentration and digestibility of plant cell-wall constituents (i.e. NDF and ADF) of which lignin is a structural and analytical component. Use of mixed-batch cultures of ruminal microorganisms in the bioassay of nutritive quality enables detection of possible effects of lignin that are not readily evident from its quantification by detergent fractionation, but to which fibrolytic ruminal microorganisms and enzyme systems might be sensitive (Powell *et al.*, 2003). *In vitro* digestibility of both the plant total DM (aerial biomass) and the cell-wall fraction (NDF) tended to decrease with increasing O_3. In previous studies, increase in foliar lignin concentration has been clearly and directly related with exposure to the pollutant (Muntifering *et al.*, 2000, 2006; Sanz *et al.*, 2005, 2011; Bender *et al.*, 2006), highlighting the importance of considering effects on quality as well as those of production when analysing O_3 effects. Results of the current study with *B. hordeaceous* are in agreement with previous observations of an inhibitory effect of O_3 on digestibility, both *in vitro* and *in vivo*, even in the absence of visible foliar injury (Powell *et al.*, 2003) or increased concentrations of detergent fibre fractions that are indicative of accelerated foliar senescence (Muntifering *et al.*, 2006; Gilliland *et al.*, 2012). The pattern of lignin response to O_3 in *B. hordeaceous* is similar to that of other grasses tested, the nutritional-quality parameters of which did not respond in a consistent manner to exposure levels to the pollutant (Gonzalez-Fernandez *et al.*, 2008). It is also important to consider that O_3 might be affecting the nutritional quality of pastures indirectly due to its ability to increase the competitiveness of the grasses. A consequent reduction in the proportion of legumes might result in the loss of digestible nutrients, the main factor that determines the quality of Mediterranean pastures (Olea and San Miguel-Ayanz, 2006). This effect could affect ingestive and processing behaviours in grazing cattle because of preferential selection of clover over grass (Rook *et al.*, 2002). Also, considering the relationship between ruminant diet and digestibility on CH_4 outputs (Misselbrook *et al.*, 2013), there may be consequences to patterns of enteric gas emissions from cattle grazing dehesa pastures.

All biomass-related parameters increased with addition of N in both assays, although the response patterns differed. In the first assay, biomass production was higher for N-medium treatments, followed by the N-high treatment. However, this pattern was not observed in the second experiment in which the N-low and N-medium treatment did not induce a significant response in *B. hordeaceous*, and only the N-high treatment stimulated its growth. Thus, the N-use efficiency was higher in the first assay because the same amount of N caused a greater increase in biomass than in the second one. Moreover, the senescent/green ratio decreased with fertilization during the first assay, whereas no effect was observed in the second. In the same way, an imbalance in the aerial/subterranean ratio caused by a greater stimulation of the aerial part was also found only in the first assay, following the common response in grasses by addition of N, just as lack of N fertilizer enhances development of the root system (Jones *et al.*, 2010). These differences in the response to N between assays could be explained by the different meteorological conditions, due to the different times when they were conducted which would have directly affected plant growth. Conditions more favourable for the development of *B. hordeaceous* during the second assay encouraged greater plant growth. In turn, greater plant growth would have required higher amounts of N to achieve the same effect on biomass percentage as that observed in the first cycle. These differences in the N effectiveness between assays might also be related to the absence of $O_3 \times N$ interaction in the second assay regarding the senescent/green biomass ratio. The results also indicate the importance of considering plant phenology linked with meteorological conditions when assessing the response of Mediterranean annual plant communities to N inputs in the system.

The long-term objective of the European Directive (2008/50/EC) and the 3-month cumulative critical level for annual communities defined by the LRTAP Convention (CLRTAP, 2010) to prevent O_3 damage to sensitive species have been set at 3000 nL L^{-1} h as a 3-month cumulative threshold value. When the O_3 effect on total biomass is considered as the response parameter, following the CLRTAP criteria, *B. hordeaceous* can be classified as a relatively O_3-tolerant species and would be protected under the currently defined thresholds. Other O_3 effects (aerial/subterranean rate, leaf hydric status), potentially giving *B. hordeaceous* some competitive advantage, would support its classification as a relatively tolerant species. This conclusion coincides with that of other annual *Poaceae* species, as analysed in previous studies, such as *Briza maxima* (Bermejo *et al.*, 2003; Sanz *et al.*, 2011). However, taking into account senescence-related parameters, *B. hordeaceous* could be classified as a species that is moderately sensitive to O_3. The exposure-response functions based on the relative response of the senescence parameters indicate that 10% of the effect was reached with O_3 exposures around 1500 nL L^{-1} h ($y = 0{\cdot}0041x + 103{\cdot}68$, $R^2 = 0{\cdot}65$ for senescent/green biomass ratio). Thus, the current critical level set for annual plant communities would not protect *B. hordeaceous* from O_3-induced effects on the senescent/green biomass ratio.

The results of this study, taken with previously published findings, indicate that species of *Poaceae* are potentially competitive with legumes and generally more sensitive to O_3 (Wilbourn *et al.*, 1995; Bermejo *et al.*, 2003; Gimeno *et al.*, 2004b) in areas subjected to chronic O_3 pollution, or in a future scenario where background O_3 levels are increased as physicochemical atmospheric models predict. From an ecological perspective, the marked difference in sensitivity to O_3 between grasses and legumes is an important risk factor for conservation of the diversity and structure of Mediterranean pasturelands. With respect to pasture nutritive quality, increasing the competitiveness of grasses vs. legumes would entail a loss of quality of Mediterranean pastures.

Conclusions

Ozone induces an increase in foliar senescence in *B. hordeaceous* with the consequent imbalance in the senescent/green biomass ratio. This response was modulated by N availability in the first assay, which mitigated the effect of the pollutant for medium O_3 levels. The intensity of this response to O_3 is related more to the O_3 absorbed fluxes inside the plant (POD) than to the atmospheric concentration of the pollutant. *Bromus hordeaceus* showed some positive responses to O_3 related to root growth and hydric content of the leaves that might give this species a competitive advantage in a high-biodiversity community with limited water and resources, such as in annual Mediterranean pastures. No significant effect on total biomass or the RFV nutritional quality index was found, although bioassay with mixed cultures of ruminal microorganisms revealed a trend towards decreased digestibility of foliar DM and cell-wall fraction associated with increased concentration of lignin.

Considering that no O_3-effects were found on green and total biomass, and some positive effects on aerial/subterranean biomass ratio or the leaf water content, *B. hordeaceus* can be classified as an O_3-tolerant species; but its sensitivity increases if senescence-related parameters are taken into account. The application of N increased biomass production in general, but intensity of response varied between experimental cycles. The greatest response to fertilizer N occurred under the growing conditions that were least favourable for the plant.

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References

Alonso R., Bermejo V., Sanz J., Valls B., Elvira S. and Gimeno B.S. (2007) Stomatal conductance of semi-natural Mediterranean grasslands: implications for the development of ozone critical levels. *Environmental Pollution*, **146**, 692–698.

Alonso R., Bermejo V., Elvira S., Aguirre Alfaro A., Sanz J., Herce Garraleta M.D., González Fernández I., Fernández Patier R. and Gimeno B.S. (2009) La contaminación atmosférica en la sierra de Guadarrama. Riesgos potenciales para la vegetación (Atmospheric pollution in the Guadarrama mountain range. Potential risks for vegetation). *VI Jornadas Científicas del Parque Natural de Peñalara y del Valle de El Paular*, pp. 63–86. Madrid: Comunidad de Madrid.

Andersen C.P. (2003) Source–sink balance and carbon allocation below ground in plants exposed to ozone. *New Phytologist*, **157**, 213–228.

Ashmore M. (2005) Assessing the future global impacts of ozone on vegetation. *Plant, Cell and Environment*, **28**, 949–964.

Association of Official Analytical Chemists. (1995) *Official methods of analysis*, 16th edn. Washington, DC: Association of Official Analytical Chemists.

Bender J., Muntifering R.B., Lin J.C. and Weigel H.J. (2006) Growth and nutritive quality of *Poa pratensis* as influenced by ozone and competition. *Environmental Pollution*, **142**, 109–115.

Bermejo V., Gimeno B.S., Sanz J., De La Torre D. and Gil J.M. (2003) Assessment of the ozone sensitivity of 22 native plant species from Mediterranean annual pastures based on visible injury. *Atmospheric Environment*, **37**, 4667–4677.

Bielenberg D.G., Lynch J.P. and Pell E.J. (2001) A decline in nitrogen availability affects plant responses to ozone. *New Phytologist*, **151**, 413–425.

Bobbink R., Hicks K., Galloway J., Spranger T., Alkemade R., Ashmore M., Bustamante M., Cinderby S., Davidson E., Dentener F., Emmett B., Erisman J.-W., Fenn M., Gilliam F., Nordin A., Pardo L. and De Vries W. (2010) Global assessment of nitrogen deposition effects on plant terrestrial biodiversity: a synthesis. *Ecological Applications*, **20**, 30–59.

Booker F., Muntifering R., McGrath M., Burkey K., Decoteau D., Fiscus E., Manning W., Krupa S., Chappelka A. and Grantz D. (2009) The ozone component of global change: potential effects on agricultural and horticultural plant yield, product quality and interactions with invasive species. *Journal of Integrative Plant Biology*, **51**, 337–351.

Buendía F. (2000) *Principales especies pascícolas de las zonas templadas (Main pasture species in temperate areas). Fundación Conde del valle de Salazar*. Ediciones Mundi Prensa: Madrid, Spain.

Calatayud V., Cerveró J., Calvo E., García-Breijo F.J., Reig-Armiñana J. and Sanz M.J. (2011) Responses of evergreen and deciduous *Quercus* species to enhanced ozone levels. *Environmental Pollution*, **159**, 55–63.

Calvo E., Martín C. and Sanz M.J. (2007) Ozone sensitivity differences in five tomato cultivars: visible injury and effects on biomass and fruits. *Water Air and Soil Pollution*, **186**, 167–181.

Castell N., Stein A.F., Salvador R., Mantilla E. and Millán M. (2008) The impact of biogenic VOC emissions on photochemical ozone formation during a high ozone pollution episode in the Iberian Peninsula in the 2003 summer season. *Advances in Science and Research*, **2**, 9–15.

CLRTAP (2010) *Manual on methodologies and criteria for modelling and mapping critical loads and levels and air pollution effects, risks and trends. Convention on Long-range Transboundary Air Pollution*. Available at:/http://www.icpmapping.org/ (accessed 17 September 2013)

Crutzen P.J., Lawrence M.G. and Poschl U. (1999) On the background photochemistry of tropospheric ozone. *Tellus*, **51**, 123–146.

Davison A.W. and Barnes J.D. (1998) Effects of ozone on wild plants. *New Phytologist*, **139**, 35–151.

Dawnay L. and Mills G. (2009) Relative effects of elevated background ozone concentrations and peak episodes on senescence and above-ground growth in four populations of *Anthoxanthum odoratum* L. *Environmental Pollution*, **157**, 503–510.

Elvira J.A., Wierman G.B., Day M.E., Greenwood M.S. and Fernandez I.J. (2006) Effects of enhanced nitrogen deposition on foliar chemistry and physiological processes of forest trees at the Bear Brook Watershed in Maine. *Forest Ecology and Management*, **221**, 207–214.

Fernandez-Fernandez M.I., Gallego M.C., Garcia J.A. and Acero F.J. (2011) A study of surface ozone variability over the Iberian Peninsula during the last fifty years. *Atmospheric Environment*, **45**, 1946–1959.

Franzaring J., Tonneijck A.E.G., Koijman A.W.N. and Dueck T.H.A. (2000) Growth responses to ozone in plant species from wetlands. *Environmental and Experimental Botany*, **44**, 39–48.

Gilliland N.J., Chappelka A.H., Muntifering R.B. and Fitzgerald L.B. (2012) Digestive utilization of ozone-exposed forage by rabbits (*Oryctolagus cuniculus*). *Environmental Pollution*, **163**, 281–286.

Gimeno B.S., Bermejo V., Reinert R., Zheng J. and Barnes J. (1999) Adverse effects of ambient ozone on watermelon yield and physiology at a rural site in eastern Spain. *New Phytologist*, **144**, 245–260.

Gimeno B.S., Bermejo V., Sanz J., De La Torre D. and Elvira S. (2004a) Growth response to ozone of annual species from Mediterranean pastures. *Environmental Pollution*, **132**, 297–306.

Gimeno B.S., Bermejo V., Sanz J., De La Torre D. and Gil J.M. (2004b) Assessment of the effects of ozone exposure and plant competition on the reproductive ability of three therophytic clover species from Iberian pastures. *Atmospheric Environment*, **38**, 2295–2303.

Gonzalez-Fernandez I., Bass D., Muntifering R., Mills G. and Barnes J.D. (2008) Impacts of ozone pollution on productivity and forage quality of grass/clover swards. *Atmospheric Environment*, **42**, 8755–8769.

González-Fernández I., Bermejo V., Alonso R., Elvira S., Sanz J. and Gimeno B.S. (2010) Modelling dehesa annual pasture dynamics: application to stomatal ozone deposition. *Atmospheric Environment*, **44**, 2507–2517.

Hauglustaine D.A. and Brasseur G.P. (2001) Evolution of tropospheric ozone under anthropogenic activities and associated radiative forcing of climate. *Journal of Geophysical Research-Atmospheres*, **106**, 32337–32360.

Hayes F., Jones M.L.M., Mills G. and Ashmore M. (2007) Meta-analysis of the relative sensitivity of semi-natural vegetation species to ozone. *Environmental Pollution*, **146**, 754–762.

Hayes F., Mills G. and Ashmore M. (2010) How much does the presence of a competitor modify the within-canopy distribution of ozone-induced senescence and visible injury? *Water Air and Soil Pollution*, **210**, 265–276.

Jarvis P.G. (1976) The interpretation of the variations in leaf water potential and stomatal conductance found in canopies in the field. *Philosophical Transactions of the Royal Society, London B*, **273**, 593–610.

Jones M.E., Paine T.D. and Fenn M.E. (2008) The effect of nitrogen additions on oak foliage and herbivore communities at sites with high and low atmospheric pollution. *Environmental Pollution*, **151**, 434–442.

Jones M.L.M., Hodges G. and Mills G. (2010) Nitrogen mediates above-ground effects of ozone but no below-ground effects in a rhizomatous sedge. *Environmental Pollution*, **158**, 559–565.

Krupa S., Muntifering R. and Chappelka A. (2004) Effects of ozone on plant nutritive quality characteristics for ruminant animals. *The Botanica*, **54**, 1–12.

Linn J.G. and Martin N.P. (1989) *Forage quality tests and interpretation*. AG-FO-02637. Minneapolis, MN, USA: University of Minnesota Extension Service.

MARM (2009) *Informe de la evaluación de la calidad del aire en España 2009 (Report on 2009 Assessment of air quality in Spain)*. Área de Calidad del Aire Subdirección General de Calidad del Aire y Medio Ambiente Industrial. Ministerio de Medio Ambiente, y Medio Rural y Marino.

Massman W.J. (1998) A review of the molecular diffusivities of H_2O, CO_2, CH_4 CO, O_3, SO_2, NH_3, N_2O, NO, and NO_2 in air, O_2 and N_2 near STP. *Atmospheric Environment*, **32**, 1111–1127.

Mickley L.J., Jacob D.J. and Rind D. (2001) Uncertainty in preindustrial abundance of tropospheric ozone: implications for radiative forcing calculations. *Journal of Geophysical Research*, **106**, 3389–3399.

Mills G., Buse A., Gimeno B., Bermejo V. and Holland M. (2007) A synthesis of AOT40-based response functions and critical levels of ozone for agricultural and horticultural crops. *Atmospheric Environment*, **41**, 2630–2643.

Misselbrook T., Del Prado A. and Chadwick D. (2013) Opportunities for reducing environmental emissions from forage-based dairy farms. *Agriculture and Food Science*, **22**, 93–107.

Muntifering R.B., Crosby D.D., Powell M.C. and Chappelka A.H. (2000) Yield and quality characteristics of bahiagrass (*Paspalum notatum*) exposed to ground-level ozone. *Animal Feed Science and Technology*, **84**, 243–256.

Muntifering R.B., Chappelka A.H., Lin J.C., Karnosky D.F. and Somers G.L. (2006) Chemical composition and digestibility of *Trifolium* exposed to elevated ozone and carbon dioxide in a free-air (FACE) fumigation system. *Functional Ecology*, **20**, 269–275.

Olea L. and San Miguel-Ayanz A. (2006) The Spanish dehesa. A traditional Mediterranean silvopastoral system linking production and nature conservation. *Grassland Science in Europe*, **11**, 1–15.

Peeters A. (2004) Wild and sown grasses. *Profiles of a temperate species selection: ecology, biodiversity and use*, pp. 117–119. Rome: FAO and Blackwell Publishing.

Pfleeger T.G., Plocher M. and Bichel P. (2010) Response of pioneer plant communities to elevated ozone exposure. *Agriculture, Ecosystems and Environment*, **138**, 116–126.

Phoenix G.K., Hicks W.K., Cinderby S., Kuylenstierna J.C.I., Stock W.D., Dentener F.J., Giller K.E., Austin A.T., Lefroy R.D.B., Gimeno B.S., Ashmore M.R. and Ineson P. (2006) Atmospheric nitrogen deposition in world biodiversity hotspots: the need for a greater global perspective in assessing N deposition impacts. *Global Change Biology*, **12**, 470–476.

Plaza J., Pujadas M. and Artiñano B. (1997) Formation and transport of the Madrid ozone plume. *Journal of Air and Waste Management Association*, **47**, 766–774.

Pleijel H., Danielsson H., Emberson L., Ashmore M.R. and Mills G. (2007) Ozone risk assessment for agricultural crops in Europe: further development of stomatal flux and flux-response relationships for European wheat and potato. *Atmospheric Environment*, **41**, 3022–3040.

Powell M.C., Muntifering R.B., Lin J.C. and Chappelka A.H. (2003) Yield nutritive quality of sericea lespedeza (*Lespedeza cuneata*) and little bluestem (*Schizachyrium scoparium*) exposed to ground-level ozone. *Environmental Pollution*, **122**, 313–322.

Rodà F., Ávila A. and Rodrigo A. (2002) Nitrogen deposition in Mediterranean forests. *Environmental Pollution*, **118**, 205–213.

Rook A.J., Harvey A., Parsons A.J., Penning P.D. and Orr R.J. (2002) Effect of long-term changes in relative resource availability on dietary preference of grazing sheep for perennial ryegrass and white clover. *Grass and Forage Science*, **57**, 54–60.

Sala O.E., Chapin F.J. III, Armesto J.J., Berlow E., Bloomfield J., Dirzo R., Huber-Sanwald E., Huenneke L., Jackson R.B., Kinzig A., Leemans R., Lodge D., Mooney H., Oesterheld M., Poff N.L., Sykes M.T., Walker B.H., Walker M. and Wall D.H. (2000) Global diversity scenarios for the year 2100. *Science*, **287**, 1770–1774.

Sanz J., Muntifering R.B., Gimeno B.S., Bermejo V. and Elvira S. (2005) Ozone and increased nitrogen supply effects on the yield and nutritive quality of *Trifolium subterraneum*. *Atmospheric Environment*, **39**, 5899–5907.

Sanz J., Bermejo V., Gimeno B.S., Elvira S. and Alonso R. (2007) Ozone sensitivity of the Mediterranean terophyte *Trifolium striatum* is modulated by soil nitrogen content. *Atmospheric Environment*, **41**, 8952–8962.

Sanz J., Bermejo V., Muntifering R., Gónzalez-Fernandez I., Gimeno B.S., Elvira S. and Alonso R. (2011) Plant phenology, growth and nutritive quality of *Briza maxima*: responses induced by enhanced ozone atmospheric levels and nitrogen enrichment. *Environmental Pollution*, **159**, 423–430.

Skärby L., Ro-Poulsen H., Wellburn F.A.M. and Sheppard L.J. (1998) Impacts of ozone on forests: a European perspective. *New Phytologist*, **139**, 109–122.

Stevens C.J., Manning P., Van Berg Den L.J.L., De Graaf M.C.C., Wieger Wamelink G.W., Boxman A.W., Bleeker A., Vergeer P., Arroniz-Crespo M., Limpens J., Lamers L.P.M., Bobbink R. and Dorland E. (2011) Ecosystem responses to reduced and oxidised nitrogen inputs in European terrestrial habitats. *Environmental Pollution*, **159**, 665–676.

Tilley J.M.A. and Terry R.A. (1963) A two-stage technique for the *in vitro* digestion of forage crops. *Journal of the British Grassland Society*, **18**, 104–111.

Van der Wal R., Pearce I., Brooker R., Scott D., Welch D. and Woodin S. (2003) Interplay between nitrogen deposition and grazing causes habit degradation. *Ecology Letters*, **6**, 141–146.

Van Soest P.J., Robertson J.B. and Lewis B.A. (1991) Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, **74**, 3583–3597.

Vazquez De Aldana B.R., Garcia-Ciudad A. and Garcia-Criado B. (2008) Interannual variations of aerial biomass and nutritional quality of Mediterranean grasslands in western Spain over a 20-year period. *Australian Journal of Agricultural Research*, **59**, 769–779.

Vollsnes A.V., Kruse O.M.O., Eriksen A.B., Oxaal U. and Futsaether C.M. (2010) In vivo root growth dynamics of ozone exposed *Trifolium subterraneum*. *Environmental and Experimental Botany*, **69**, 183–188.

Wilbourn S., Davidson A.W. and Ollerenshaw J.H. (1995) The use of an unenclosed field fumigation system to determine the effects of elevated ozone on a grass-clover mixture. *New Phytologist*, **129**, 23–32.

Wyness K., Mills G., Jones L., Barnes J.J. and Ones D. (2011) Enhanced nitrogen deposition exacerbates the negative effect of increasing background ozone in *Dactylis glomerata*, but not *Ranunculus acris*. *Environmental Pollution*, **159**, 2493–2499.

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Ozone and nitrogen effects on yield and nutritive quality of the annual legume *Trifolium cherleri*

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HIGHLIGHTS

- Ozone reduced the aerial and subterranean biomass of the annual legume.
- Ozone-induced senescence in *Trifolium cherleri* can be enhanced by N supplementation.
- Ozone increased the foliar lignin content diminishing the quality of the pasture.
- The O_3-response was not correlated to the ozone fluxes inside the plants.

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ABSTRACT

Two independent experiments were performed in an Open-Top Chamber facility to determine the response of biomass and nutritive quality of the annual legume *Trifolium cherleri* to increased levels of ozone (O_3) and nitrogen (N) deposition, two main drivers of global change. Plants growing in pots were exposed to three O_3 treatments: charcoal-filtered air (CFA); non-filtered air, reproducing ambient O_3 levels of the site (NFA); and non-filtered air supplemented with 40 nl l^{-1} (NFA+). Nitrogen was added in biweekly doses to achieve final doses of 5 (N5), 15 (N15) and 30 kg ha^{-1} (N30), reproducing the N deposition range in the Iberian Peninsula.

Ozone negatively affected all the growth-related parameters and increased plant senescent biomass. The pollutant affected subterranean biomass to a greater extent than aerial biomass, resulting in altered aerial/subterranean ratio. Effects in the second experiment followed the same pattern as in the first, but were of lesser magnitude. However, these differences between assays could not be explained adequately by the absorbed O_3 fluxes (Phytotoxic Ozone Dose, POD). Concentrations of cell-wall constituents related to nutritive quality increased with the O_3 exposure, reducing the Relative Food Value index (RFV) that indicates decreased nutritive quality of the forage.

Nitrogen stimulated all growth-related parameters, but increased the aboveground biomass more than the subterranean biomass. No effects of N fertilizer were detected for the nutritive quality parameters. A significant interaction between O_3 and N was found in the second experiment. N further enhanced the increase of senescent biomass caused by O_3.

Results indicate that O_3 is a potentially significant environmental stress factor in terms of structure and diversity of Mediterranean pastures.

1. Introduction

Ozone (O_3) occurs naturally in the atmosphere at varying concentrations, depending on the altitude. Due its physical and chemical properties, changes in its concentration can have implications for the biosphere. In the troposphere O_3 contributes to the greenhouse gas effect and its high oxidizing capacity can negatively affect biological organisms. Background levels in the troposphere

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have doubled since the beginning of the Industrial Revolution in the mid-Nineteenth Century up to the present day for which the current, predicted annual increase is 0.5–2.0% (Vingarzan, 2004). This increase is due to anthropogenic emissions of O_3 precursors which, under stable atmospheric conditions at high temperatures and through a series of photochemical reactions, are responsible for the formation of the pollutant (Guicherit and Roemer, 2000). However, because of the complexity of interactions between the precursor reactions and the atmosphere, simply controlling emissions is not sufficient to produce a significant reduction in O_3 levels (Langner et al., 2005). It is predicted that O_3 concentrations in the Mediterranean area will continue to increase throughout this century, despite restrictions imposed within the European Union to control O_3 precursor emissions resulting from increasing temperatures, drier summers and reduced cloud coverage (Meleux et al., 2007). In the Iberian Peninsula, the critical levels established by legislation to protect the vegetation are presently being exceeded (MAGRAMA, 2013). The adverse effects that O_3 can cause to European pastures (which represent approximately 50% of land usage in Europe) are of great importance in terms of biodiversity, production and food sources in livestock farming (Krupa et al., 2004). A wide range of susceptibilities have been found in herbaceous pasture species. Leguminous plants are the least tolerant, which could affect the competition between species and lead to changes in the composition of pastures (Gimeno et al., 2004b; Hayes et al., 2009). Ozone effects also include, but are not limited to, decreased biomass production (Gimeno et al., 2004a), acceleration of senescence (Dawnay and Mills, 2009), altered distribution of assimilated carbon (Vollsnes et al., 2010) and changes in cell wall structures, particularly an increase in lignin content (Sanz et al., 2005; González-Fernández et al., 2008).

The use of fossil fuels in industry and transport, coupled with the intensification of agricultural systems, is responsible for the emission of large volumes of both oxidized and reduced forms of nitrogen (N) into the atmosphere, converting immense quantities of stable atmospheric nitrogen (N_2) into reactive forms, and in turn causing changes to the N cycle and a cascade of environmental problems (ENA, 2011). Increased N deposition in ecosystems poses risks for plant communities because it can lead to a loss in diversity (Bobbink et al., 2010). Changes in species richness caused by N deposition have been discovered in the acidic grasslands of Northern Europe (Stevens et al., 2004, 2006; Duprè et al., 2010). In regard to Mediterranean ecosystems, almost all of the studies investigating the impact of N deposition have been conducted in California by means of fertilization experiments in the field and gradient studies (Allen et al., 1998; Fenn et al., 2010). Very few studies have been conducted in the Mediterranean Basin itself, but surprisingly the results obtained differ from the majority of the studies performed in California and Northern Europe. Dias et al. (2011) observed an increase in the diversity of vascular plants, explained by the increased N availability in soil that was extremely deficient in nutrients. However, Bonanomi et al. (2009), studying a semi-natural calcareous grassland, did not detect any changes in diversity when the soil N was enriched. Due to the variety of responses discovered, new studies are necessary in Mediterranean ecosystems so that the effect of N deposition in the ground and its influence on vegetation can be evaluated.

The coexistence of elevated levels of O_3 plus N deposition can alter the equilibrium of ecosystems (Takemoto et al., 2001). In the mixed-conifer forest of the San Bernardino Mountains (California) where elevated levels of O_3 coincide with elevated N deposition, a decrease in the biomass of fine-tree roots (Grulke et al., 1998), changes in C and N concentrations, and an alteration in water balance were observed, increasing the ecosystem's susceptibility to biotic and abiotic stresses.

In the interior of the Iberian Peninsula, large areas of dehesa are exposed to a combination of elevated O_3 levels and N deposition (Alonso et al., 2009). The dehesa is an agroforestry system formed by open holm oak forests. Their understory pasture is primarily composed of herbaceous annuals, with a high seasonal and annual variability in both production and quality, which provide food for wildlife and livestock (Vazquez de Aldana et al., 2008). Different studies have been performed to evaluate the repercussions of O_3 and N on these herbaceous communities (Bermejo et al., 2003; Gimeno et al., 2004a,b; Sanz et al., 2005, 2007, 2011), particularly in species belonging to the legume and grass families because of their importance in terms of number of species and their nutritive value for grazing livestock (Marañon, 1985). The results showed that the legumes, particularly the *Trifolium* genus, were more susceptible to O_3, which could influence the competitiveness of these species and affect the structure, diversity and quality of Mediterranean pastures. Legume species are important components for pastures due to their role as N fixers and their nutritive quality for herbivores (Vázquez de Aldana et al., 2008). However, this functional group shows a considerable variability in its sensitivity to O_3 (Gimeno et al., 2004a). More information on the response of individual species is required to evaluate the potential effects of ozone at the community level.

The present study conducted on *Trifolium cherleri* aims to provide more information required to understand the potential changes in Mediterranean herbaceous communities caused by the increased levels of O_3 and N deposition, two of the main drivers of the global change in the area. On the view of previous studies, it is hypothesized that the clover might be O_3 sensitive but the response to the pollutant would be modulated by the N availability.

2. Materials and methods

The experimental studies were performed using an Open-Top chamber (OTC) facility located in a rural zone distant from any sources of pollution in the northeast of the Iberian Peninsula (Tarragona, Spain; 40° 41′ N, 0° 47′ E). A sequential, automatic system provided continuous measures of the O_3, SO_2, NO and NO_2 concentrations inside each of the chambers. For a detailed description of the experimental facility, refer to Alonso et al. (2001). The closest weather station of the Catalonian Meteorological Service to the field site was used to acquire air temperature, relative humidity and solar radiation values (Fangar, Tarragona, Spain; 40° 47′ N, 0° 46′ E). Two independent experiments were conducted using the same design, for which the calendar of events is detailed in Table 1.

Table 1
Timetable of events for the *Trifolium cherleri* experiments.

Event	Date	DaS
First experiment		
Sowing	27 May	
Emergence	29 May	
N fertilization	29 June	
Transplantation	1 July	
Start of ozone exposure in OTCs	2 July	0
N fertilization	15 July	13
Harvest	31 July	29
Second experiment		
Sowing	19 August	
Transplantation	30 September	
Start of ozone exposure in OTCs	1 October	0
N fertilization	2 October	1
N fertilization	21 October	20
End of OTC exposure	12 November	43
Harvest	12 November	43

DaS (Days after start of experiment).

2.1. Plant material

Seeds were supplied by the germplasm bank of the Department of Agriculture and Environment of the Autonomous Community of Extremadura (Spain). Seed origin was from a natural population of *T. cherleri* in a dehesa located in the west of the Iberian Peninsula (38° 22′ N, 0° 64′ W). The seeds were submerged in a solution of Germinator© (Agro-orgánicos Mediterráneos S.L., Granada, Spain) for 24 h to stimulate and homogenize the germination. Next, those seeds that swelled were sown in seed-tray compartments containing a mixed substrate of 50% vermiculite and 50% white peat. Approximately one month later, the seedlings were transplanted into 2.5-L pots containing a mixed substrate of 50% peat, 20% vermiculite and 30% perlite, to which $CaCO_3$ was added to adjust the pH to six. Approximately 10 cm^3 of soil, collected from a dehesa to the north of Madrid inhabited by *T. cherleri*, was added to each pot to promote root nodulation. All of the nutrients required for balanced plant growth were supplied by means of a nutrient solution prepared with a water-soluble fertilizer of low N content (Peters, N-P-K: 4-25-35 plus microelements). Throughout the experiment, water was fed to the pots via a drip irrigation system, ensuring a continuous and adequate availability of water to the plants.

2.2. Ozone and nitrogen treatments

During the first and second experiments, plants were exposed for 4 and 6 weeks, respectively, to three O_3 treatments: carbon-filtered air without O_3 (CFA), non-filtered air that reproduces the ambient O_3 concentrations (NFA), and non-filtered air plus 40 nl l^{-1} of added O_3 (NFA+). Plants were exposed to the pollutant for five consecutive days a week from 07:00 to 17:00 h (GMT). The O_3 exposure was expressed in terms of the accumulated AOT40 index that is currently used by the European legislation (2008/50/EC) and the Convention on Long-range Transboundary Air Pollution (CLRTAP, 2010) for evaluating the O_3 risk to vegetation. It is calculated from the sum of the hourly average of O_3 concentrations in excess of 40 nl l^{-1} during the growing period. Three replicate chambers were used per O_3 treatment and three plants per N treatment within each OTC (9 plants per N and O_3 treatment). Nitrogen was applied in two additional doses, using different NH_4NO_3 (35%) solutions, to obtain three total N treatments of 5 (N5), 15 (N15) and 30 (N30) kg N ha^{-1}.

2.3. Biomass parameters

At the end of the O_3 exposure, above-ground biomass was collected in a single harvest by cutting flush with the substrate, distinguishing between green and senescent biomass. Visible foliar injuries caused by O_3 were evaluated on two randomly selected stalks per plant, counting the number of leaves with signs of injury in relation to the total number of leaves. Roots were carefully extracted by continually washing with water. Root nodules were observed in all the plants and treatments, although they were not quantified nor their activity evaluated. The above- and below-ground biomass was dried to constant weight at 60 °C. The senescent/green and above-ground/below-ground biomass ratios were calculated.

2.4. Nutritive quality parameters

Nutritive quality was only analysed in for the summer experiment. The crude protein content ($CP = \% N \times 6.25$) was determined by the Kjeldhal method (Association of Official Analytical Chemists, 1995). The concentrations of acid and neutral detergent fibre fractions (ADF and NDF, respectively) and lignin were determined sequentially according to the procedures described by Van Soest et al. (1991). Calculation of RFV (Relative Food Value, Linn and Martin, 1989) was based on foliar ADF and NDF concentrations. The green biomass from three pots per chamber and the N treatment were composited for the quality analysis.

Table 2
Mean, maximum and minimum daily meteorological values and AOT40 index, accumulated over the fumigation period, for the *Trifolium cherleri* assays. RH = air relative humidity; SR = total solar radiation; VPD = atmospheric water vapour pressure deficit; CFA = Charcoal filtered air; NFA = Non-filtered air; NFA+ = Non filtered air +40 nl l^{-1} O_3.

	Mean	Max.	Min.
First experiment			
Temperature (°C)	24.6	32.0	17.7
RH (%)	83	100	30
SR (Wm^{-2})	271	948	0
VPD (Kpa)	0.57	3.04	0
AOT40 (nl l^{-1} h) CFA	39		
AOT40 (nl l^{-1} h) NFA	1198		
AOT40 (nl l^{-1} h) NFA+	10190		
Second experiment			
Temperature (°C)	18.4	25.6	11.9
RH (%)	84	100	51
SR (Wm^{-2})	142	750	0
VPD (Kpa)	0.36	1.52	0
AOT40 (nl l^{-1} h) CFA	0		
AOT40 (nl l^{-1} h) NFA	221		
AOT40 (nl l^{-1} h) NFA+	6032		

2.5. Statistical analysis

The effects of O_3 and N supplementation were analysed via a split-plot ANOVA analysis. The Tukey test was employed to separate whenever the ANOVA analysis indicated the presence of significant differences among treatments ($p < 0.05$). Prior to the ANOVA analysis, the variance homogeneity was checked using the Levene test and the normality of the variables and their residuals using the corresponding graphs. A log transformation was performed on the variables that did not comply with any of the above requirements.

2.6. Calculation of Phytotoxic Ozone Dose

Ozone stomatal fluxes, or phytotoxic O_3 dose (POD), were calculated following the criteria developed under the Convention on Long-range Transboundary Air Pollution (CLRTAP, 2010). Environmental conditions inside the chambers were estimated using data from Fangar meteorological station and the relationships between data inside and outside the chambers obtained in previous experiments in the same facility. Stomatal conductance was estimated using a multiplicative model (Jarvis, 1976) parameterized for *Trifolium subterraneum* by González-Fernández et al. (2010) because no parameterization was available for *T. cherleri*. In the stomatal conductance model, f_{phen} and f_{SWP} were set to 1, and g_{max} was transformed to mmol O_3 m^{-2} s^{-1} using the coefficient of diffusivity factors of H_2O and O_3 in air, 0.663 (Massman, 1998). POD was accumulated from the start of the O_3 fumigation treatments until the end of the experiment. No flux thresholds were considered for the calculation of the total POD.

3. Results and discussion

3.1. Meteorological conditions and ozone levels

The O_3 values recorded during both experiments were within the seasonal and interannual variability that this pollutant can reach in the Iberian Peninsula (Fernández-Fernández et al., 2011). The AOT40 values obtained for O_3 treatments, including mean and extreme values of meteorological parameters for both experiments, are presented in Table 2. Atmospheric conditions varied between the two experiments. The mean VPD value for the first experiment (0.55 kPa) was higher than in the second experiment (0.36 kPa) because the average temperature was 6 °C higher in the former, whereas the relative humidity values were similar for both (70–90%). The number of hours and intensity of solar radiation were also higher in the first experiment.

Ozone concentrations also varied across the experiments. The O_3 concentrations in the OTCs were higher in the first experiment due to the higher O_3 levels in the ambient air (Table 2), due to higher temperatures and solar radiation during this period. In the first experiment, an AOT40 value of 1198 nl l^{-1} h was registered in ambient air as compared with 221 nl l^{-1}h in the second experiment. Exposure to O_3 in the NFA treatment during the first experiment, if applied for three months, would have been sufficient to cause damage to the most susceptible species for which AOT40-response relations have been determined (Mills et al., 2007).

3.2. Visible foliar injury

O_3 exposure induced visible symptoms on leaves of plants grown under NFA and NFA+ treatments. The symptoms observed were reddish-brown necrotic spots, similar to those typically found in this species and in others of the *Trifolium* genus that are characteristic of Mediterranean pastures (Bermejo et al., 2003). The percentages of damaged leaves by the same O_3 treatment were in close proximity for both experiments, although slightly higher in the second: 14% and 19% for the first and second experiments, respectively, in the NFA treatment; and 57% and 39% for the NFA+ treatment. A previous study with *T. cherleri* show a higher percentage of affected leaves, 32% and 60% associated with AOT40 values of 783 and 15 493 nl l^{-1} h respectively (Bermejo et al., 2003). The variations in the response to O_3 between the different studies might be related to prevailing meteorological conditions as they affect the gas-exchange process. It is well known that the O_3 injury is related more to O_3 absorbed through the stomatal pores than to the air concentration values (González-Fernández et al., 2010). Accordingly, under the most favourable conditions for gas exchange observed in the second experiment, visible symptoms in *T. cherleri* were observed in almost 20% of the leaves with only a 1.5 month-AOT40 of 221 nl l^{-1} h. However, the more growth-limiting conditions of the first experiment, in spite of the higher O_3 values, caused a lesser severity of foliar symptoms. Based on the development of visible injury in this clover and by comparing with results described in other studies (Bermejo et al., 2003), we can categorize this species as being O_3-sensitive.

3.3. Biomass

In the first experiment, O_3 induced a significant reduction in the green, total aerial, subterranean and total biomass parameters ($p < 0.009$) and an increase in the senescent biomass ($p < 0.02$), although differences were only significant between NFA+ and the control CFA (Table 3). The green biomass decreased by 47% for NFA+ compared with CFA across all N treatments, whereas the senescent biomass in NFA+ increased by 100%, resulting in an increase in the senescent/green biomass ratio of 250%. In the NFA treatment that reproduced ambient levels, this ratio increased by only 21% as a result of the 11% loss in green biomass. The total above-ground biomass (sum of green and senescent) in NFA+ decreased by an average of 33% across all N treatments compared with the CFA control ($p < 0.02$), whereas a non-significant 10% biomass reduction was observed in plants grown in NFA (Table 3). The ozone exposure also affected the root growth ($p < 0.0004$), inducing reductions in subterranean biomass of 14 and 43% in NFA and NFA+ compared with CFA. The greater O_3 sensitivity of the root system compared with the aerial resulted in a no statistically significant 25% mean increase in the above-ground/below-ground ratio between plants subjected to the upper levels of O_3 exposure (NFA+) (Table 3). Total biomass (sum of the above and below-ground biomass) decreased by 11 and 37% in NFA and NFA+ compared with the filtered air control respectively (Table 3).

The effect of O_3 was considerably less in the second experiment (Table 4). The O_3 treatment non significantly decreased the green biomass by 16% in NFA+ compared with CFA across all N treatments. O_3 strongly enhanced the senescent biomass in NFA+ with respect to both NFA and CFA ($p < 0.1$) by an average of 207%, which caused the senescent/green biomass ratio to increase by an average of 226% in the NFA+ compared with NFA and CFA.

The results obtained, primarily from the first experiment, are similar to results published in a previous screening study by Gimeno et al. (2004a). In this screening *T. cherleri* displayed a moderate susceptibility to O_3 compared with other annual legumes in which the above-ground biomass reduction was associated with an increase in senescent biomass and appearance of visible injury. Another fairly common response in O_3 sensitive plants is a greater effect in the root system than in the above-ground biomass (Wyness et al., 2011; Vollsnes et al., 2010). This can lead to an increase in the above-ground/below-ground biomass ratio reflecting an altered carbon allocation in the plant (Andersen, 2003). A decrease in root biomass and an increase in the above-ground/below-ground biomass ratio have also been observed in other Mediterranean annual species from the *Trifolium* genus (Sanz et al., 2005, 2007). A reduction in the subterranean biomass can have important ecological and pastoral consequences because it could affect the duration of the plant phenological cycle and its competitiveness in a high-diversity pasture with a limited access to nutrients and soil moisture characteristic of the Mediterranean environments (Gallardo et al., 2009). In coping with these factors, annual pastures are characterised by high spatial and temporal complementarity among their many component species (Cleland et al., 2006) in such a way that small changes in a given species can lead into major changes in the structure and diversity of the community.

In both experiments, increased N in the substrate resulted in a stimulation of all biomass parameters except senescent biomass, and the effect was stronger in the second than in the first experiment (Tables 3 and 4). Green biomass and total above-ground biomass increased significantly and progressively with increasing N-supply. Green biomass in the first experiment was 32% and 77% higher for N15 and N30 treatments, respectively, compared with N5 ($p < 0.0001$) (mean across O_3 treatments); in the second experiment, the same comparisons yielded differences of 41% and 92% ($p < 0.0001$). Total above-ground biomass increased in the first and second experiments, respectively, were 28% and 37% for N15, and 63% and 87% for N30. Although the N-induced effect on senescence was not statistically significant, the senescent/green ratio decreased as the N dose increased. Extent of N-induced reduction ($p < 0.0001$) in senescent/green ratio was similar in both trials: 15% and 22% for N15 and 45% and 33% for N30 in the first and second experiments respectively. Nitrogen stimulated root growth, but to a lesser extent compared with aboveground biomass ($p < 0.0001$). Subterranean biomass increased under N30 by a mean percentage of 36% and 69% in the first and second experiments respectively, compared with N5 and N15 across all O_3 treatments. The difference in the response between aerial and subterranean biomass unbalanced the above-ground/below-

Table 3
Growth-related parameters (plant means se) corresponding to the exposure of *Trifolium cherleri* to the different O_3 and N treatments on the first experiment.

First experiment	Green biomass (g dw)	Senescent biomass (g dw)	Senes./green biomass ratio	Total aerial biomass (g dw)	Subt. biomass (g dw)	Total biomass (g dw)	Aerial/Subt. ratio
O_3	<0.009	<0.02	<0.0003	<0.02	<0.0004	<0.006	ns
Nitrogen	<0.0001	ns	<0.0001	<0.0001	<0.0001	<0.0001	<0.002
O_3*Nitrogen	ns	ns	ns	ns	ns	ns	ns
Low-N CFA	0.70 ± 0.07	0.07 ± 0.01	0.12 ± 0.02	0.78 ± 0.07	0.49 ± 0.05	1.27 ± 0.11	1.59 ± 0.08
Low-N NFA	0.55 ± 0.08	0.11 ± 0.01	0.21 ± 0.03	0.66 ± 0.08	0.38 ± 0.05	1.04 ± 0.13	1.79 ± 0.11
Low-N NFA+	0.36 ± 0.04	0.17 ± 0.01	0.48 ± 0.05	0.53 ± 0.04	0.26 ± 0.03	0.79 ± 0.08	2.18 ± 0.17
Medium-N CFA	0.85 ± 0.09	0.10 ± 0.01	0.13 ± 0.02	0.95 ± 0.08	0.49 ± 0.04	1.45 ± 0.12	1.96 ± 0.10
Medium-N NFA	0.77 ± 0.07	0.09 ± 0.01	0.13 ± 0.02	0.86 ± 0.07	0.46 ± 0.04	1.32 ± 0.11	1.87 ± 0.08
Medium-N NFA+	0.48 ± 0.04	0.20 ± 0.02	0.43 ± 0.04	0.69 ± 0.06	0.31 ± 0.04	1.00 ± 0.10	2.34 ± 0.16
High-N CFA	1.15 ± 0.06	0.10 ± 0.01	0.09 ± 0.01	1.25 ± 0.07	0.66 ± 0.07	1.91 ± 0.13	2.01 ± 0.14
High-N NFA	1.09 ± 0.10	0.07 ± 0.01	0.07 ± 0.01	1.16 ± 0.10	0.57 ± 0.06	1.74 ± 0.16	2.10 ± 0.13
High-N NFA+	0.60 ± 0.05	0.17 ± 0.03	0.28 ± 0.03	0.77 ± 0.07	0.36 ± 0.06	1.13 ± 0.13	2.42 ± 0.21

The *p* values corresponding to the effect of each factor (O_3 and N) are presented in the first part of the table, whereas treatment means se are presented below. CFA = charcoal filtered air; NFA = non-filtered air; NFA+ = non filtered air +40 ppb O_3; Low, Medium and High-N represent N supply at 5, 15 and 30 kg ha^{-1}; respectively.

ground ratio ($p < 0.02$), bringing to an increment in this rate: N30 enhanced by 29% and 17% for the first and second experiments respectively compared with the lower dose. Total biomass increased progressively with increasing N dose ($p < 0.0001$). Significant increases were found in N15 (22%) and N30 (54%) compared to N5 in the first experiment and in N30 (73%) compared with N5.

In the second experiment, a significant interaction was observed for senescent biomass ($p < 0.0001$). Increasing N fertilizer amplified the effects caused by the high levels of the pollutant such that medium and high doses of N enhanced the O_3-induced increase in senescent biomass caused by exposure to the NFA+ treatment. It is generally accepted that the mineral status of plants can alter their responses to O_3 (Davison and Barnes, 1998); however, published results report different conclusions. Bassin et al. (2007) reported a response with a subalpine grassland that was similar to that in the present study, in contrast to results found for the grasses *Briza maxima* (Sanz et al., 2011) and *Carex arenaria* (Jones et al., 2010) where increasing N availability reduced the O_3-induced increment in senescence biomass. Consistent with these latter reports, N partially counteracted the increase in senescent/green biomass ratio caused by exposure to elevated O_3 concentrations in *T. subterraneum* (Sanz et al., 2005) and in *B.maxima* (Sanz et al., 2011). Very interesting results were also found with *Trifolium striatum* for which Sanz et al. (2007) showed how N fertilizer can counterbalance the loss of flowers and seeds induced by moderate levels of O_3. The effects of O_3 and N on plants are highly complex and appear to be modulated by several factors including concentrations, species, parameters assessed and prevailing environmental factors (Bassin et al., 2007; Ochoa-Hueso et al., 2011). There is no clear mechanistic understanding of the interactions between these two factors in the literature (Davison and Barnes, 1998) and no general conclusions could be derived from this study. Further investigation would be needed to ascertain the potential interactive effects between O_3 and N.

The two experiments with *T. cherleri* showed different responses to O_3 and N levels. The effects of O_3 exposure were more pronounced in the first experiment, whereas the effects of N were greater in the second. N increased total biomass by 22 and 54% in the medium and high N treatments with respect to the low N treatment in the first experiment. In the second experiment these increases were as high as 30 and 73%. Differences in ambient O_3 concentrations, atmospheric conditions and exposure periods may have resulted in differences of O_3 doses which are regulated by the stomatal pore opening. In an attempt to explain the different O_3-response observed between the exposure periods, the AOT40 and the POD were estimated.

The stronger plant response to O_3 during the first experiment correlates adequately with the increase in recorded O_3 levels for both experimental periods considering an accumulated O_3 index: the AOT40 levels for the NFA and NFA+ treatments were 1198 nl l^{-1} and 10 891 nl l^{-1}, respectively, being far higher than the levels of 221 nl l^{-1} and 6032 nl l^{-1} recorded in the second experiment. The experimental setting in this study does not consider important environmental variables like soil moisture that can limit stomatal deposition of O_3 under field conditions. Thus, effects presented here should be interpreted as a worst case scenario for each given O_3 exposure. POD estimates that consider soil moisture and meteorological limitations to stomatal conductance may be more adequate for estimating the risk of O_3 negative effects. The estimated POD was slightly lower in the first experiment (Table 5). Although this observation is in accordance with the limitations imposed by the meteorological conditions for stomatal opening during this first period, it is contradictory with the higher O_3 effects found in this experiment. This apparent disagreement between fluxes and effects might suggest a need to incorporate into the POD estimation the biochemical mechanisms responsible for detoxification and repair from oxidative stress in plants exposed to O_3 (Castagna and Ranieri, 2009). Such detoxification processes are also subject to different activity associated with ambient conditions such as temperature, radiation or water stress (Heath et al., 2009). This variability can contribute to a non-linearity between ozone exposure and effects (Heath et al., 2009) that may explain the differences observed on treatment effects between the first and second experiments. The identification and quantification of the processes responsible for cellular detoxification and repair in plants exposed to O_3 present major difficulties and was not measured in this study; however, it is currently considered a determining factor for improvement of O_3 plant-risk assessment (Tuzet et al., 2011).

Effects of N treatment were more pronounced in the second than the first experiment. This difference also appears to be indicative of an interaction between the treatment and the more favourable meteorological conditions of the second experiment, producing bigger plants at the same levels of N supplementation. Various studies have shown that environmental variables affecting the primary production of vegetation can regulate the response of Mediterranean species and ecosystems to N (Ochoa-Hueso et al., 2011).

3.4. Nutritive quality

Ozone caused significant effects on all of the nutritive quality parameters analysed ($p < 0.01$), whereas N only affected the crude protein (CP) concentration ($p < 0.05$) (Table 6).

Table 4
Growth-related parameters (plant means se) corresponding to the exposure of *Trifolium cherleri* to the different O_3 and N treatments on the second experiment.

Second experiment	Green biomass (g dw)	Senescent biomass (g dw)	Senes./green biomass ratio	Total aerial biomass (g dw)	Subt. biomass (g dw)	Total biomass (g dw)	Aerial/Subt. Ratio
O_3	ns	<0.1	<0.03	ns	ns	ns	ns
Nitrogen	<0.0001	ns	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
O_3*Nitrogen	ns	<0.0001	ns	ns	ns	ns	ns
Low-N CFA	1.16 ± 0.13	0.08 ± 0.02^{ab}	0.07 ± 0.00	1.24 ± 0.15	0.65 ± 0.07	1.89 ± 0.21	1.88 ± 0.05
Low-N NFA	1.14 ± 0.10	0.07 ± 0.03^{a}	0.06 ± 0.02	1.21 ± 0.10	0.57 ± 0.05	1.79 ± 0.13	2.24 ± 0.31
Low-N NFA+	0.90 ± 0.14	0.11 ± 0.02^{ab}	0.14 ± 0.02	1.01 ± 0.15	0.51 ± 0.05	1.51 ± 0.19	1.96 ± 0.18
Medium-N CFA	1.49 ± 0.16	0.04 ± 0.01^{a}	0.03 ± 0.01	1.52 ± 0.17	0.66 ± 0.05	2.18 ± 0.21	2.30 ± 0.14
Medium-N NFA	1.73 ± 0.26	0.06 ± 0.03^{a}	0.06 ± 0.03	1.80 ± 0.25	0.68 ± 0.06	2.48 ± 0.30	2.65 ± 0.36
Medium-N NFA+	1.28 ± 0.17	0.16 ± 0.02^{b}	0.13 ± 0.01	1.44 ± 0.19	0.62 ± 0.05	2.07 ± 0.23	2.30 ± 0.19
High-N CFA	2.02 ± 0.18	0.04 ± 0.01^{a}	0.02 ± 0.00	2.07 ± 0.19	0.78 ± 0.06	2.85 ± 0.24	2.66 ± 0.16
High-N NFA	2.34 ± 0.15	0.05 ± 0.02^{a}	0.02 ± 0.00	2.38 ± 0.17	0.89 ± 0.09	3.27 ± 0.22	2.89 ± 0.31
High-N NFA+	1.76 ± 0.14	0.25 ± 0.03^{c}	0.14 ± 0.01	2.01 ± 0.16	0.85 ± 0.05	2.85 ± 0.16	2.42 ± 0.22

The *p* values corresponding to the effect of each factor (O_3 and N) are presented in the first part of the table, whereas treatment means se are presented below. CFA = charcoal filtered air; NFA = non-filtered air; NFA+ = non filtered air +40 ppb O_3; Low, Medium and High-N represent N supply at 5, 15 and 30 kg ha^{-1}; respectively. Different letters indicate significant differences among means in each N treatment when interactive effects were significant.

Table 5
Phytotoxic Ozone Dose (POD) accumulated from the start of the O_3 exposure until the end of the harvest for the different O_3 treatments. CFA = Charcoal filtered air; NFA = Non-filtered air; NFA+ = Non filtered air +40 nl l^{-1} O_3. No thresholds were considered for the calculation of the total POD (POD0).

Trifolium cherleri	1st experiment			2nd experiment		
	CFA	NFA	NFA+	CFA	NFA	NFA+
POD_0 (mmol O_3 m^{-2} s^{-1})	2.5	11.1	19.3	3.0	16.7	27.4

Foliar CP was augmented by increasing O_3 exposure and N dose, with no interaction between these factors. The CP concentration in plants was increased by 17% and 36% for NFA and NFA+ treatments, respectively, compared with CFA across all N treatments). Values for N15 and N30 doses were increased by 19% and 27%, respectively, compared with N5 across all O_3 treatments. Differences among treatments were only significant when comparing the highest level of each factor with their corresponding controls. However, this positive effect on foliar CP concentration is counterbalanced by the overall yield reduction caused by O_3, which would result in a distinct loss of protein yield of the pasture. Protein yield was reduced by O_3 ($p = 0.07$) by 28.1% in NFA+ compared with CFA but it slightly increased by 6.5% in NFA. A similar response in CP was observed in the annual legume *T. subterraneum* (Sanz et al., 2005), but differs from the response of the annual grass *B. maxima* whose CP was not altered by N dose, and for which only a slight increase was induced by the O_3-exposure (Sanz et al., 2011). The O_3 effects on CP concentration in forages have also been described in other studies based on herbaceous perennials such as the grasses *Paspalum notatum* (Muntifering et al., 2000) and *Schizachyrium scoparium* (Powell et al., 2003). The CP increase in these species could be related to biomass decrease and a concentration effect (Piikki et al., 2008), although it could also have resulted from an alteration of the plant N metabolism (Davison and Barnes, 1998).

Exposure to O_3 also affected foliar concentrations of the NDF, ADF and lignin fractions. The NDF concentration was significantly increased by 6% in plants under NFA+ treatment compared with NFA and CFA ($p < 0.001$) across N treatments. The increase in ADF concentration was also 6%, but the difference was only statistically significant between the NFA+ and NFA treatments. The lignin concentrations in plants grown under O_3 exposure showed an extraordinary increase of 49% in NFA (although non-significant) and 150% under the most elevated level of O_3 (NFA+) compared with the control CFA ($p < 0.001$). The increase in the concentrations of cell wall constituents reflected in the proportions of NDF, ADF and lignin has a negative impact on the free-range voluntary intake and forage digestibility (Muntifering et al., 2006). In agreement with the results obtained, the nutritive quality index RFV decreased significantly by 7% in plants grown in NFA+ compared with those grown in less contaminated CFA and NFA environments. There were no significant effects on nutritive quality expressed as RFV caused by ambient O_3 levels recorded during the development of the assay.

The increase in concentrations of cell wall constituents induced by O_3 has been described previously in a large number of herbaceous species (Krupa et al., 2004). A similar but more pronounced response was observed in *T. subterraneum*, which showed a significant increase in the foliar fibre concentration in plants grown at lower levels of an NFA treatment (Sanz et al., 2005). Ozone also increased the NDF and ADF concentrations in the more O_3-resistant annual grass *B. maxima*, even when no response was found for other biomass parameters (Sanz et al., 2011), which illustrates the high sensitivity of nutritive–quality parameters to the pollutant. Other studies with forage perennials species such as the legumes *Medicago sativa* (Lin et al., 2007), *Trifolium pratense* or *Trifolium repens* (Muntifering et al., 2006) displayed a greater susceptibility to O_3 than perennial grasses species such as *P. notatum* (Muntifering

Table 6
Ozone (O_3) and nitrogen supply (N) effects on nutritive quality parameters of *Trifolium cherleri* at the end of the exposure period (30 days).

First assay	CP (%)	NDF (%)	ADF (%)	Lignin (%)	RFV
O_3	<0.04	<0.04	<0.03	<0.002	<0.05
Nitrogen	<0.03	ns	ns	ns	ns
O_3*Nitrogen	ns	ns	ns	ns	ns
N-5 CFA	8.3 ± 0.2	36.5 ± 1.0	23.5 ± 0.6	0.6 ± 0.1	180 ± 6
N-15 CFA	8.5 ± 1.1	37.3 ± 0.3	23.9 ± 0.2	0.4 ± 0.1	175 ± 1
N-30 CFA	9.4 ± 0.4	37.4 ± 0.8	24.0 ± 0.3	0.6 ± 0.1	174 ± 4
N-5 NFA	8.5 ± 0.6	37.1 ± 0.9	22.7 ± 0.4	0.7 ± 0.1	178 ± 5
N-15 NFA	10.5 ± 0.1	37.1 ± 0.7	23.1 ± 0.4	0.8 ± 0.1	178 ± 4
N-30 NFA	11.6 ± 0.6	37.5 ± 0.6	23.2 ± 0.6	1.1 ± 0.1	175 ± 4
N-5 NFA+	9.9 ± 1.0	39.1 ± 0.5	23.7 ± 0.2	1.5 ± 0.2	167 ± 3
N-15 NFA+	12.8 ± 1.6	40.2 ± 0.3	25.0 ± 0.1	1.5 ± 0.1	160 ± 1
N-30 NFA+	13.1 ± 1.5	38.9 ± 0.4	24.2 ± 0.5	1.6 ± 0.1	167 ± 2

The upper part of the table presents results of the two-way ANOVA that was conducted to evaluate effects of O_3 and N, singly and in combination, on the assessed parameters. The lower part of the table presents mean values and their standard errors corresponding to the different treatments. CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; RFV = relative feed value (standardized by reference to a medium-quality forage containing 53% NDF, 41% ADF and RFV of 100); CFA = Charcoal filtered air; NFA = Non-filtered air; NFA+ = Non filtered air + 40 ppb O_3; Low-N = total N supply of 5 kg ha^{-1} y^{-1};Medium-N = total N supply of 15 kg ha^{-1} y^{-1}; High-N = total N supply of 30 kg ha^{-1} y^{-1}.

et al., 2000) or *Poa pratensis* (Bender et al., 2006). These results based on quality parameters support the agronomic generalization that legumes are more O_3-sensitive than grasses, as usually happens when biomass parameters are considered.

The increase in lignin content of cell walls is a common response of plants to different stress factors, both biotic and abiotic, most notably elevated levels of O_3 (Cabane et al., 2012). Ozone increases the activity of various enzymes (PAL, CHS, CAD) that control the biosynthesis of flavonoids and phenylpropanoids, precursors of lignin formation (Castagna and Ranieri, 2009). In previous studies performed on species ecologically close to *T. cherleri* such as *B. maxima* and *T. subterraneum*, O_3 increased the lignin concentrations to a greater or lesser extent, with the susceptibility of *T. subterraneum* standing out with increases of 200% and 366% in NFA and NFA+ treatments, respectively, compared with CFA (Sanz et al., 2005). The increase in the concentration of fibres and lignin in the cell walls of herbaceous annuals is related to aging and flowering onset (Lloveras and Iglesias, 2001). In *B. maxima* and *T. striatum*, O_3 altered plant phenology, advancing flowering and reducing the life-cycle, which coincided with an increase in the foliar fibre concentration at the end of the exposure period (Sanz et al., 2011, 2007). Nitrogen fertilization was not able to counteract the negative effects of O_3 on the forage nutritive quality.

4. Conclusions

The response of *T. cherleri* to O_3 involved the appearance of visible foliar symptoms associated with a decline in the production of above-ground biomass and increase in senescent biomass. Treatment effects were more important in the subterranean biomass. Combined with these effects, nutritive–quality parameters were sensitive to the pollutant, showing an increase in the lignin concentration and other cell wall components (possibly related to an accelerated life-cycle) and thus negatively influence the quantity and quality of *T. cherleri* as sustenance for wild herbivores and livestock.

Results point to O_3 as a stress factor for the structure and biodiversity of the Mediterranean pastures.

Nitrogen supplementation, at levels simulating the N deposition values found in the Iberian Peninsula, stimulated biomass parameters, but caused no effects on the nutritive quality parameters. Nitrogen fertilizer can enhance the negative effects of O_3 in terms of the production of senescent biomass.

Differences found in the response of the legume to the pollutant between assays cannot be adequately explained by the O_3 absorbed fluxes (Phototoxic Ozone Dose, POD), but results highlight the importance of the meteorological conditions on the response to O_3 and N.

Considering the appearance of specific foliar symptoms, *T. cherleri* can be classified as an O_3-sensitive species, but when effects on biomass production and nutritive quality parameters are considered it should be classified as moderately-sensitive to the pollutant.

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References

Alonso, R., Elvira, S., Castillo, F.J., Gimeno, B.S., 2001. Interactive effects of ozone and drought stress on pigments and activities of oxidative enzymes in *Pinus halepensis*. Plant Cell Environ. 24, 905–916.

Alonso, R., Bermejo, V., Elvira, S., Aguirre Alfaro, A., Sanz, J., Herce Garraleta, M.D., González Fernández, I., Fernández Patier, R., Gimeno, B.S., 2009. La contaminación atmosférica en la sierra de Guadarrama. Riesgos potenciales la vegetación. In: VI Jornadas Científicas del Parque Natural de Peñalara y del Valle de El Paular. Comunidad de Madrid, pp. 63–86.

Andersen, C.P., 2003. Source–sink balance and carbon allocation below ground in plants exposed to ozone. New. Phytol. 157, 213–228.

Allen, E.B., Padgett, P.E., Bytnerowicz, A., Minnich, R.A., 1998. Nitrogen deposition effects on coastal sage vegetation of southern California. In: Proceedings of the International Symposium on Air Pollution and Climate Change Effects on Forest Ecosystems. US Department of Agriculture Forest Service, Pacific Southwest Research Station, Riverside, California, USA, pp. 131–140.

Bassin, S., Volk, M., Suter, M., Buchmann, N., Fuhrer, J., 2007. Nitrogen deposition but not ozone affects productivity and community composition of subalpine grassland after 3 yr of treatment. New. Phytol. 175, 523–534.

Bender, J., Muntifering, R.B., Lin, J.C., Weigel, H.J., 2006. Growth and nutritive quality of *Poa pratensis* as influenced by ozone and competition. Environ. Pollut. 142, 109–115.

Bermejo, V., Gimeno, B.S., Sanz, J., de la Torre, D., Gil, J.M., 2003. Assessment of the ozone sensitivity of 22 native plant species from Mediterranean annual pastures based on visible injury. Atmos. Environ. 37, 4667–4677.

Bobbink, R., Hicks, K., Galloway, J., Spranger, T., Alkemade, R., Ashmore, M., Bustamante, M., Cinderby, S., Davidson, E., Dentener, F., Emmett, B., Erisman, J.-W., Fenn, M., Gilliam, F., Nordin, A., Pardo, L., de Vries, W., 2010. Global assessment of nitrogen deposition effects on plant terrestrial biodiversity: a synthesis. Ecol. Appl. 20, 30–59.

Bonanomi, G., Caporaso, S., Allegrezza, M., 2009. Effects of nitrogen enrichment, plant litter removal and cutting on a species-rich Mediterranean calcareous grassland. Plant Biosyst. 143, 443–455.

Cabane, M., Afif, D., Hawkins, S., 2012. Lignins and abiotic stresses. Adv. Bot. Res. 61, 219–262.

Castagna, A., Ranieri, A., 2009. Detoxification and repair process of ozone injury: from O_3 uptake to gene expression adjustment. Environ. Pollut. 157, 1461–1469.

Cleland, E.E., Chiariello, N.R., Loarie, S.R., Mooney, H.A., Field, C.B., 2006. Diverse responses of phenology to global changes in a grassland ecosystem. Proc. Natl. Acad. Sci. 103, 13740–13744.

CLRTAP (Convention on Long-Range Transboundary Air Pollution), 2010. Manual on Methodologies and Criteria for Modelling and Mapping Critical Loads and Levels and Air Pollution Effects, Risks and Trends. http://www.icpmapping.org/.

Davison, A.W., Barnes, J.D., 1998. Effects of ozone on wild plants. New. Phytol. 139, 35–151.

Dawnay, L., Mills, G., 2009. Relative effects of elevated background ozone concentrations and peak episodes on senescence and above-ground growth in four populations of *Anthoxanthum odoratum* L. Environ. Pollut. 157, 503–510.

Dias, T., Malveiro, S., Martins-Loução, M.A., Sheppard, L., Cruz, C., 2011. Linking increased N-driven biodiversity changes with soil N availability in a mediterranean-type ecosystem. Plant Soil 341, 125–136.

Duprè, C., Stevens, C.J., Ranke, T., Bleeker, A., Peppler-Lisbach, C., Gowing, D.J.G., Dise, N.B., Dorland, E., Bobbink, R., Diekmann, M., 2010. Changes in species richness and composition in European acidic grasslands over the past 70 years: the contribution of cumulative atmospheric nitrogen deposition. Glob. Change Biol. 16, 344–357.

ENA (European Nitrogen Assessment), 2011. In: Sutton, M.A., Howard, C.M., Erisman, J.W. (Eds.), The European Nitrogen Assessment. Sources, Effects and Policy Perspectives. Cambridge University Press, Cambridge.

Fenn, M.E., Allen, E.B., Weiss, S.B., Jovan, S., Geiser, L., Tonnesen, G.S., Johnson, R.F., Rao, L.E., Gimeno, B.S., Yuan, F., Meixner, T., Bytnerowicz, A., 2010. Nitrogen critical loads and management alternatives for N-impacted ecosystems in California. J. Environ. Manag. 91, 2404–2423.

Fernández-Fernández, M.I., Gallego, M.C., Garcia, J.A., Acero, F.J., 2011. A study of surface ozone variability over the Iberian Peninsula during the last fifty years. Atmos. Environ. 45, 1946–1959.

Gallardo, A., Covelo, F., Morillas, L., Delgado, M., 2009. Nutrient cycling and soil processes in terrestrial ecosystems: specific nature of Mediterranean ecosystem and its implications for the soil–plant relationship. Ecosistemas 18, 4–19.

Gimeno, B.S., Bermejo, V., Sanz, J., de la Torre, D., Elvira, S., 2004a. Growth response to ozone of annual species from Mediterranean pastures. Environ. Pollut. 132, 297–306.

Gimeno, B.S., Bermejo, V., Sanz, J., de la Torre, D., Gil, J.M., 2004b. Assessment of the effects of ozone exposure and plant competition on the reproductive ability of three therophytic clover species from Iberian pastures. Atmos. Environ. 38, 2295–2303.

González-Fernández, I., Bass, D., Muntifering, R., Mills, G., Barnes, J., 2008. Impacts of ozone pollution on productivity and forage quality of grass/clover swards. Atmos. Environ. 42, 8755–8769.

González-Fernández, I., Bermejo, V., Alonso, R., Elvira, S., Sanz, J., Gimeno, B.S., 2010. Modelling dehesa annual pasture dynamics: application to stomatal ozone deposition. Atmos. Environ. 44, 2507–2517.

Grulke, N.E., Andersen, C.P., Fenn, M.E., Miller, P.R., 1998. Ozone exposure and nitrogen deposition lowers root biomass of ponderosa pine in the San Bernardino Mountains, California. Environ. Pollut. 103, 63–73.

Guicherit, R., Roemer, M., 2000. Tropospheric ozone trends. Chemosphere: Global Change Science 2, 167–183.

Hayes, F., Mills, G., Ashmore, M., 2009. Effects of ozone on inter- and intra-species competition and photosynthesis in mesocosms of *Lolium perenne* and *Trifolium repens*. Environ. Pollut. 157, 208–214.

Heath, R.L., Lefohn, A.S., Musselman, R.C., 2009. Temporal processes that contribute to nonlinearity in vegetation responses to ozone exposure and dose. Atmos. Environ. 43, 2919–2928.

Jarvis, P.G., 1976. The interpretation of the variations in leaf water potential and stomatal conductance found in canopies in the field. Philos. Trans. R. Soc. Lond. B 273, 593–610.

Jones, M.L.M., Hodges, G., Mills, G., 2010. Nitrogen mediates above-ground effects of ozone but no below-ground effects in a rhizomatous sedge. Environ. Pollut. 158, 559–565.

Krupa, S., Muntifering, R., Chappelka, A., 2004. Effects of ozone on plant nutritive quality characteristics for ruminant animals. Botanica 54, 1–12.

Langner, L., Bergström, R., Foltescu, V., 2005. Impact of climate change on surface ozone and deposition of sulphur and nitrogen in Europe. Atmos. Environ. 39, 1129–1141.

Linn, J.G., Martin, N.P., 1989. Forage Quality Tests and Interpretation. AG-FO-02637. University of Minnesota Extension Service, Minneapolis.

Lin, J.C., Nosal, M., Muntifering, R.B., Krupa, S.V., 2007. Alfalfa nutritive quality for ruminant livestock as influenced by ambient air quality in west-central Alberta. Environ. Pollut. 149, 99–103.

Lloveras, J., Iglesias, I., 2001. Morphological development and forage quality changes in crimson clover (*Trifolium incarnatum* L.). Grass Forage Sci. 56, 395–404.

MAGRAMA (Ministry of Agriculture, Food and Environment of Spain), 2013. Informe de la evaluación de la calidad del aire en España 2012. Dirección General de Calidad y Evaluación Ambiental y Medio Natural. Subdirección General de Calidad del Aire y Medio Ambiente Industrial. Ministerio de Agricultura, Alimentación y Medio Ambiente.

Massman, W.J., 1998. A review of the molecular diffusivities of H_2O, CO_2, CH_4, CO, O_3, SO_2, NH_3, N_2O, NO, and NO_2 in air, O_2 and N_2 near STP. Atmos. Environ. 32, 1111–1127.

Meleux, F., Solmon, F., Giorgi, F., 2007. Increase in summer European ozone amounts due to climate change. Atmos. Environ. 41, 7577–7587.

Marañon, T., 1985. Diversidad florística y heterogeneidad ambiental en una dehesa de Sierra Morena. An. Edafol. Agrobiol. 44, 1183–1197.

Mills, G., Buse, A., Gimeno, B., Bermejo, V., Holland, M., 2007. A synthesis of AOT40-based response functions and critical levels of ozone for agricultural and horticultural crops. Atmos. Environ. 41, 2630–2643.

Muntifering, R.B., Crosby, D.D., Powel, M.C., Chappelka, A.H., 2000. Yield and quality characteristics of bahiagrass (*Paspalum notatum*) exposed to ground-level ozone. Animal Feed Sci. Technol. 84, 243–256.

Muntifering, R.B., Chappelka, A.H., Lin, Karnosky, D.F., Somers, G.L., 2006. Chemical composition and digestibility of *Trifolium* exposed to elevated ozone and carbon dioxide in a free-air (FACE) fumigation system. Funct. Ecol. 20, 269–275.

Ochoa-Hueso, R., Allen, E.B., Branquinho, C., Cruz, C., Dias, T., Fenn, M.E., Manrique, E., Stock, W.D., 2011. Nitrogen deposition effects on Mediterranean-type ecosystems: an ecological assessment. Environ. Pollut. 159, 2265–2279.

Piikki, K., De Temmerman, L., Ojamperä, K., Danielsson, H., Pleijel, H., 2008. The grain quality of spring wheat (*Triticum aestivum* L.) in relation to elevated ozone uptake and carbon dioxide exposure. Eur. J. Agronomy 28, 245–254.

Powell, M.C., Muntifering, R.B., Lin, J.C., Chappelka, A.H., 2003. Yield nutritive quality of seriacea lespedeza (*Lespedeza cuneata*) and little bluestem (*Schizachyrium scoparium*) exposed to ground-level ozone. Environ. Pollut. 122, 313–322.

Sanz, J., Muntifering, R.B., Gimeno, B.S., Bermejo, V., Elvira, S., 2005. Ozone and increased nitrogen supply effects on the yield and nutritive quality of *Trifolium subterraneum*. Atmos. Environ. 39, 5899–5907.

Sanz, J., Bermejo, V., Gimeno, B.S., Elvira, S., Alonso, R., 2007. Ozone sensitivity of the Mediterranean terophyte *Trifolium striatum* is modulated by soil nitrogen content. Atmos. Environ. 41, 8952–8962.

Sanz, J., Bermejo, V., Muntifering, R., Gónzalez-Fernandez, I., Gimeno, B.S., Elvira, S., Alonso, R., 2011. Plant phenology, growth and nutritive quality of *Briza maxima*: responses induced by enhanced ozone atmospheric levels and nitrogen enrichment. Environ. Pollut. 159, 423–430.

Stevens, C.J., Dise, N.B., Mountford, J.O., Gowing, D.J., 2004. Impact of nitrogen deposition on the species richness of grasslands. Science 303, 1876–1879.

Stevens, C.J., Dise, N.B., Gowing, D.J., Mountford, J.O., 2006. Loss of forb diversity in relation to nitrogen deposition in the UK: regional trends and potential controls. Glob. Change Biol. 12, 1823–1833.

Takemoto, B.K., Bytnerowicz, A., Fenn, M.E., 2001. Current and future effects of ozone and atmospheric nitrogen deposition on California's mixed conifer forests. For. Ecol. Manag. 144, 159–173.

Tuzet, A., Perrier, A., Loubet, B., Cellier, P., 2011. Modelling ozone deposition fluxes: the relative roles of deposition and detoxification processes. Agric. For. Meteorol. 151, 480–492.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597.

Vazquez-de-Aldana, B.R., Garcia-Ciudad, A., Garcia-Criado, 2008. Interannual variations of aboveground biomass and nutritional quality of Mediterranean grasslands in Western Spain over a 20-year period. Aust. J. Agric. Res. 59, 769–779.

Vingarzan, R., 2004. A review of surface ozone background levels and trends. Atmos. Environ. 38, 3431–3442.

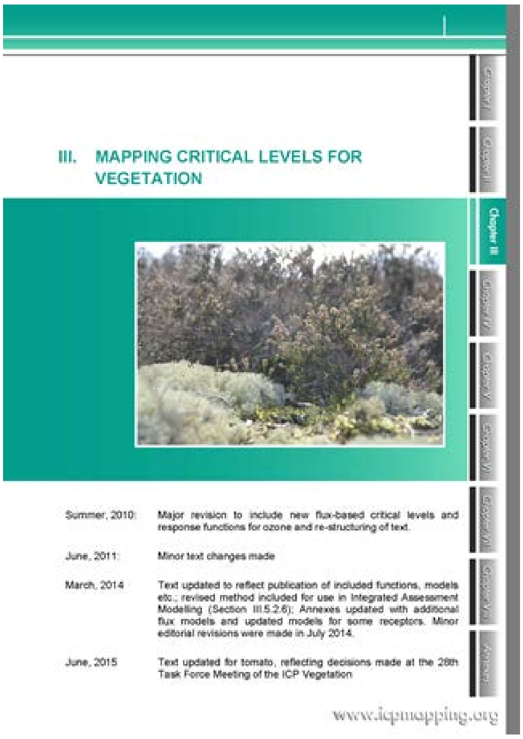
Vollsnes, A.V., Kruse, O.M.O., Eriksen, A.B., Oxaal, U., Futsaether, C.M., 2010. In vivo root growth dynamics of ozone exposed *Trifolium subterraneum*. Environ. Exp. Bot. 69, 183–188.

Wyness, K., Mills, G., Jones, L., Barnes, J., Jones, D., 2011. Enhanced nitrogen deposition exacerbates the negative effect of increasing background ozone in *Dactylis glomerata*, but not *Ranunculus acris*. Environ. Pollut. 159, 2493–2499.

3.4. PROPUESTA DE NIVELES CRÍTICOS DE OZONO EN ESPECIES PASCÍCOLAS MEDITERRÁNEAS ANUALES

Se analiza de forma conjunta los efectos del O_3 en herbáceas anuales mediterráneas a partir de cinco experimentos previos desarrollados en un campo experimental de cámaras de techo descubierto (OTCs), con el objetivo de calcular las funciones generales de exposición y dosis-respuesta al O_3. Se revisan y proponen nuevos niveles críticos siguiendo la metodología del CLRTAP/ENECE (CLRTAP, 2010).

Cuando el diseño experimental incluyó otros factores además del O_3, como la disponibilidad de N o el factor competencia, se ha considerado en el análisis solo la respuesta al contaminante promedio entre niveles de N o grados de competencia por cada tratamiento de O_3. Los niveles críticos propuestos se han calculado en base a las especies sensibles al O_3 para cada parámetro, por lo que el empleo de las funciones de exposición y dosis-respuesta obtenidas deberían utilizarse para análisis de riesgo, no para cuantificación de daños.



III. MAPPING CRITICAL LEVELS FOR VEGETATION

Summer, 2010:	Major revision to include new flux-based critical levels and response functions for ozone and re-structuring of text.
June, 2011:	Minor text changes made
March, 2014	Text updated to reflect publication of included functions, models etc.; revised method included for use in Integrated Assessment Modelling (Section III.5.2.6); Annexes updated with additional flux models and updated models for some receptors. Minor editorial revisions were made in July 2014.
June, 2015	Text updated for tomato, reflecting decisions made at the 28th Task Force Meeting of the ICP Vegetation

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Figura 13. Manual del CLRTAP (2015) donde se describe la metodología para el cálculo de los niveles críticos de O_3 para los distintos tipos de vegetación.

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Setting ozone critical levels for annual Mediterranean pasture species: Combined analysis of open-top chamber experiments

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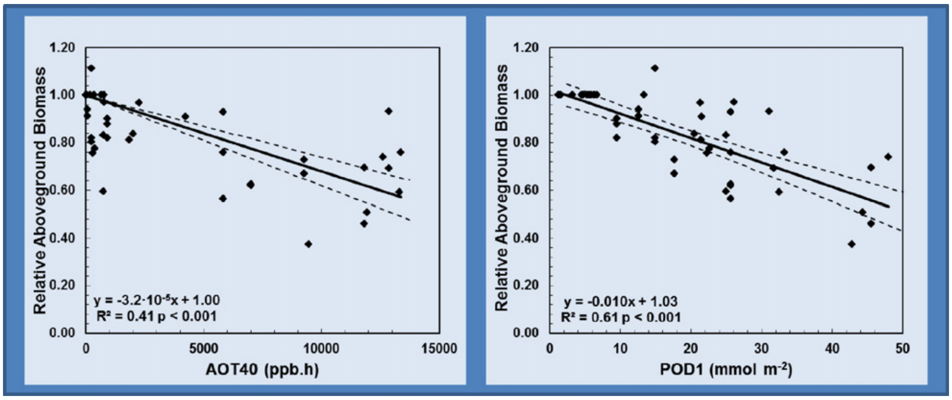
HIGHLIGHTS

- O_3 critical levels are developed for annual Mediterranean pasture species.
- Functions available for aboveground biomass, consumable food value and seed biomass.
- Functions were derived only for sensitive species.
- O_3 flux indices performed better than the AOT40.
- Functions valid only for risk assessment not suitable for quantification of effects

GRAPHICAL ABSTRACT



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ABSTRACT

Annual Dehesa-type pastures comprise semi-natural vegetation communities dominated by annual species characteristic of the Mediterranean basin areas of Southern Europe. This study analyses all the datasets available on the effects of ozone (O_3) on annual pasture species in order to review and propose new exposure- and flux-based O_3 critical levels (CLes) following the methodology of the Convention on Long-Range Transboundary Air Pollution (CLRTAP). Based on the potential effect on pastures main ecosystem services, the availability of data and the statistical significance of the regressions, three variables have been selected for establishing CLes: total above-ground biomass, consumable food value (CFV), as a nutritional quality index, and reproductive capacity based on flower and seed production. New CLes proposed for a 10% loss (with 95% confidence intervals between brackets) of above-ground biomass and reproductive capacity were, respectively, AOT40 = 3.1 (2.6, 3.8) and 2.0 (1.5, 2.8) ppm h and POD1 = 12.2 (8.9, 15.5) and 7.2 (1.1, 13.3) mmol m^{-2}. The provisional AOT40- and POD1-based CLes for CFV were 2.3 (1.6, 4.0) ppm h and 4.6 (2.7, 6.5) mmol m^{-2} respectively. By using only O_3-sensitive species for the exposure and dose-response functions, the proposed CLes should be used for risk assessments. Their use for quantifying O_3 damage may lead to an overestimation of the effects.

1. Introduction

Gas emissions from human activities into the atmosphere have substantially changed its composition, causing increased concentrations of tropospheric ozone (O_3) (Vingarzan, 2004). This pollutant is a powerful

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oxidant that induces damage to living organisms (Weschler, 2006; Lenka and Lenka, 2012). During the late fifties in California, in areas with air masses polluted by atmospheric oxidants, small, brown to black, discrete, punctate lesions appearing on the upper part of vine leaves (Richards et al., 1958) and flecking on tobacco leaves (Heggestad and Middleton, 1959) were identified. This was the first evidence of the toxic nature of O_3 on vegetation. Since then visible damage has been detected in a large number of plant species distributed through a sizeable number of countries (Mills et al., 2011a), serving to verify the importance of the toxic effects of O_3 on vegetation. The effects of O_3 on agricultural crops and forest trees have been extensively studied and documented, however, on herbaceous vegetation they are less known (CLRTAP, 2010) due to the number of species and the complexity of their communities (Caballero et al., 2009).

The Dehesa, a traditional agro-silvopastoral system comprising an open oak forest with annual pasture understory, occupies $>6\cdot10^6$ ha in the Iberian peninsula. Its ecological functioning is conditioned by two fundamental characteristics, the Mediterranean climate and a low soil fertility (Olea and San Miguel-Ayanz, 2006). Today it is protected by the Habitat Directive 92/43/EEC (Annex 1 habitat 6310) and included in the Natura 2000 network, as an example of the sustainable management of natural resources. Dehesas hold different herbaceous communities, mainly composed by annual species providing fodder for wildlife and livestock. Their great annual and seasonal variability, in terms of production and nutritional quality, is related with the variability of the climatic conditions of the area (Vazquez-de-Aldana et al., 2008). Besides, these pastures present a high floristic diversity which ranges 102–135 species per 0.1 ha^{-1}, with the most prevalent families being *Gramineae*, *Leguminosae* and *Compositae* (Marañon, 1985; García del Barrio et al., 2014). Inter-annual meteorological variability characteristic of the Mediterranean climate can bring early droughts leading to plants dying even before producing seeds, but in more humid years the life span can extend until early summer. Therefore, annuals have evolved developing transient and persistent seed banks. Seed germination is diversified depending on species, soil moisture, temperature and light and it can even be partially delayed until more favourable years (Peco et al., 2009). This leads to a characteristic inter-annual change in the species composition of the pasture (Vazquez-de-Aldana et al., 2008).

In the past decade, several studies have been conducted to evaluate the O_3 effects in Mediterranean herbaceous communities within the dehesas. Results indicated some legume genus like *Trifolium* among the most O_3 sensitive but others tolerant like *Ornithopus* or *Anthyllis* (Gimeno et al., 2004a), showing not only a high variability between but also within families. Therefore, the heterogeneous response of the species to the pollutant might change species competitiveness affecting the biodiversity and nutritional quality of the pastures (Calvete-Sogo et al., 2016). Some of the O_3 effects described for sensitive annuals include: the appearance of visible characteristic symptoms (Chaudhary and Agrawal, 2013); decreasing growth rate (Gimeno et al., 2004a); senescence acceleration (Bermejo et al., 2003); altered assimilated carbon allocation (Sanz et al., 2005); reduced seed production (Gimeno et al., 2004b; Sanz et al., 2007); changes in the structure of cell walls, in particular the increase in the lignin content (Sanz et al., 2014); loss of forage quality (Sanz et al., 2014); and modification of the phenological cycle usually shortening the growing season (Sanz et al., 2011).

Tropospheric O_3 is currently considered as the most phytotoxic air pollutant given the large number of sensitive plant species and the geographic extent of the problem. Accordingly, this pollutant has been included in international policies and programmes to develop environmental management strategies which control air pollution like the UNECE Convention on Long-Range Transboundary Air Pollution (CLRTAP). The CLRTAP developed the methodology of Critical Levels to establish environmental criteria based on the sensitivity of vegetation to O_3 and other gaseous pollutants to negotiate protocols for reducing atmospheric emissions.

Critical levels (CLe) of O_3 were first established in 1996 and have been revised periodically based on new knowledge (Mills et al., 2011b). Today CLe for vegetation are defined as: "concentration, cumulative exposure or cumulative stomatal flux of atmospheric pollutants above which direct adverse effects on sensitive vegetation may occur according to present knowledge". The CLe are calculated using exposure and dose-response relationships using O_3 concentration-based indices like the AOT40, which is the exposure index currently used by the European legislation on air quality (2008/50/EC), or O_3 flux-based indices (POD_y) more recently developed under the frame of the CLRTAP. However, some important limitations and uncertainties have been recognized when AOT40 is used. Primarily, because the impact depends on the amount of O_3 absorbed by the leaves, while this index considers the O_3 concentration in the air at canopy height. As a result, current studies focus on developing stomatal flux-based CLe (CLe_f), as plant responses are generally more closely related to the O_3 dose absorbed through the stomata than to O_3 exposure (Mills et al., 2011b; González-Fernandez et al., 2014).

This study analyses the available data sets from studies conducted on Mediterranean Dehesa annual herbaceous species following a similar experimental protocol, open-top chamber (OTC) experiments and plants growing in pots, with the aim of reviewing and proposing new O_3 CLe for this type of vegetation under the framework of the CLRTAP (2010).

2. Materials and methods

2.1. Open-top chamber experiments

Data were analysed from five independent experiments in an OTC experimental field located in a rural area in the northeast of the Iberian Peninsula (Tarragona, 40° 41′ N, 0° 47′ E), away from local sources of air pollution. For a detailed description of the experimental facility see Alonso et al. (2001). The experiments were conducted over four campaigns from 2000 to 2003. The therophyte species assayed belonged to the *Leguminoseae* (10 species) and *Gramineae* (9 species) families, two of the most prevalent families in Mediterranean grasslands of high grazing and ecological value (Vazquez-de-Aldana et al., 2008). All studies followed a similar protocol: plants were grown in pots on similar artificial substrate and were kept at field water capacity. The O_3 exposure length was similar, 45 days on average. Filtered air treatments (CFA) were used on all experiments and considered as the control treatment. In some experiments the plants were exposed to other factors such as soil N availability and plant-plant competition in order to observe possible interactions with the O_3. However, interactions between factors are not discussed herein. Ozone effects were analysed considering the average effect across the levels of the non-O_3 factors. Further details regarding the species, exposure levels and dates, parameters measured, factor assayed and related references are indicated in Table 1.

2.2. Response parameters and ozone indices

Response parameters studied can be classified into three categories: biomass, grazing quality and reproductive capacity. Biomass parameters studied were: aerial green, senescent, total aerial (sum of green and senescent biomass), subterranean and total biomass (sum of aboveground and subterranean biomass). The ratios senescent/green biomass and total aerial/subterranean biomass were also calculated. Nutritional quality parameters from the sequential separation of cell wall constituents according to the method of Van Soest et al. (1991) were studied: the Neutral Detergent Fibre (NDF) fraction containing insoluble constituents of the cell wall (hemicellulose, cellulose, lignin and other recalcitrant materials); the Acid Detergent Fibre (ADF) fraction, consisting of lignocellulosic material and other recalcitrant materials; and the Acid Detergent Lignin (ADL) fraction, consisting of lignin and other recalcitrant materials. Two indices of nutritional quality were also calculated considering these parameters: The relative food value (RFV), which integrates the NDF and ADF (Linn and Martin, 1989), and the consumable food value (CFV), which integrates NDF and ADF with the green biomass loss (González-Fernández et al., 2008). Given the strong correlation

Table 1
Experiments included in the study. CFA = charcoal filtered air; NFA = Non filtered air; NFA+ = Non filtered air supplemented with O_3.

Year	Species	Dates for POD calculation	Days POD accumulated	O_3 treatment	Other factors	Variables	References
2000	*Anthylliscornicina*	07/09–22/11	76	CFA, NFA, NFA+	Monocultures	Visible injury Senescence Aerial and subterranean biomass	Bermejo et al. (2003) Gimeno et al. (2004a)
	Anthyllislotoides	14/09–22/11	69				
	Biserrulapelecinus	22/09–27/11	67				
	Medicago minima	11/10–22/11	43				
	Ornithopuscompressus	11/10–27/11	47				
	Trifoliumangustifolium	11/10–23/11	43				
	Trifoliumcherleri	08/09–08/11	61				
	Trifoliumglomeratum	08/09–08/11	61				
	Trifolium striatum	14/09–13/11	54				
	Trifoliumsubterraneum	08/09–12/11	65				
	Aegilopsgeniculata	07/09–10/11	64				
	Aegilopstriuncialis	07/09–11/11	65				
	Avena sterilis	03/10–21/11	49				
	Briza maxima	20/09–22/11	63				
	Bromushordeaceus	07/09–16/11	70				
	Bromussterilis	14/09–20/11	67				
	Cynosurusechinatus	14/09–21/11	68				
	Loliumrigidum	11/10–23/11	43				
	Vulpiamyuros	20/09–22/11	63				
2001	*Trifoliumcherleri*	28/05–12/07	45	CFA, NFA, NFA+	Competition	Aerial biomass	Unpublished results
	Trifolium striatum	30/05–13/07	44				
	Trifoliumsubterraneum	31/05–17/07	47				
	Ornithopuscompressus	29/05–18/07	50				
	Briza maxima	29/05–17/07	49				
	Bromushordeaceus	30/05–20/07	51				
	Trifoliumcherleri	28/05–08/08	72	CFA, NFA, NFA+	Competition	Flower production Seed production (final harvests from previous experiment)	Gimeno et al. (2004b)
		28/05–08/09	103				
	Trifolium striatum	30/05–14/07	45				
		30/05–13/08	75				
		30/05–05/09	98				
	Trifoliumsubterraneum	31/05–18/08	79				
2002	*Trifoliumcherleri*	02/07–31/07	29	CFA, NFA, NFA+	Nitrogen	Aerial and subterranean biomass Forage quality	Sanz et al. (2014)
	Trifolium striatum	02/07–31/07	29			Phenology Aerial and subterranean biomass Forage quality	Sanz et al. (2007)
	Trifoliumsubterraneum	02/07–31/07	29			Aerial and subterranean biomass Forage quality	Sanz et al. (2005)
	Briza maxima	02/07–07/08	36			Phenology Aerial and subterranean biomass Forage quality	Sanz et al. (2011)
	Bromushordeaceus	02/07–07/08	36			Aerial and subterranean biomass Forage quality	Sanz et al. (2013)
	Cynosurusechinatus	02/07–07/08	36			Aerial and subterranean biomass Forage quality	Unpublished results
	Trifolium striatum	02/07–06/09	66	CFA	Nitrogen	Seed production (final harvest of previous experiment)	Sanz et al. (2007)
		02/07–02/09	62	NFA			
		02/07–26/08	55	NFA+			
	Trifoliumcherleri	01/10–12/11	42	CFA, NFA, NFA+	Nitrogen	Aerial and subterranean biomass	Sanz et al. (2014)
	Trifolium striatum	01/10–12/11	42				Unpublished results
	Trifoliumsubterraneum	01/10–12/11	42				Unpublished results
	Briza maxima	01/10–12/11	42				Sanz et al. (2011)
	Bromushordeaceus	01/10–12/11	42				Sanz et al. (2013)
	Cynosurusechinatus	01/10–12/11	42				Unpublished results
2003	*Trifolium striatum*	15/05–25/06	36	CFA, NFA, NFA+	Nitrogen × Competition	Aerial biomass	Unpublished results
	Maximum Briza	15/05–25/06	36			Phenology	
	Trifolium striatum	15/05–15/07	61	CFA	Nitrogen × Competition	Seed production (final harvest of previous experiment)	Unpublished results
		15/05–14/07	60	NFA			
		15/05–11/07	57	NFA+			

between the seed weight and the flower weight for the O_3 sensitive annuals (Gimeno et al., 2004b) both were joined to evaluate the O_3 effects on the reproductive capacity.

The O_3 indices tested were calculated following the CLRTAP methodology (CLRTAP, 2010). The AOT40 exposure index was calculated as the hourly accumulated concentration above 40 ppb during daylight hours (solar radiation > 50 W m^{-2}). Stomatal O_3 flux or O_3 phytotoxic dose (POD_y) was based on the modelled stomatal conductance, detailed below, using different flux thresholds (0, 1, 3, and 6 nmol m^{-2} s^{-1}) to identify the most suitable index for predicting the O_3-induced effects. The accumulation period of AOT40 and POD_y for biomass and quality parameters coincided with the exposure period inside the OTCs: from the start of the exposure until harvesting, averaging 45 days. However, two accumulation periods were considered for the reproductive capacity: 1 - from the start of the O_3 exposure until biomass harvesting (45 days average); and 2 - from the start of exposure until seed maturity (71 days average).

2.3. Stomatal conductance

Stomatal conductance was modelled using a multiplicative approach (Jarvis, 1976) using the only two currently available parameterizations for the assayed families. *Leguminosae* species were based on the parameterization of *Trifolium subterraneum* by González-Fernández et al. (2010), while *Gramineae*, was based on the *Bromus hordeaceus* parameterization (Alonso et al., 2007). The maximum stomatal conductance (g_{max}) of *Bromus hordeaceus* has been modified to include the field values recorded for this species during two measurement campaigns in 2005 and 2007 (González-Fernández et al., 2010). In the stomatal conductance model the phenological function (f_{phen}) and the soil water potential function (f_{SWP}) were set to 1, and g_{max} was transformed to mmol O_3 m^{-2} s^{-1} using the coefficient of molecular diffusivity between H_2O and O_3 in air of 0.663 (Massman, 1998).

2.4. Linear correlation analysis and ozone response functions

The O_3 effects for the different parameters and experiments were expressed as percentages relative to the CFA treatment. The critical level concept is by definition aimed at protecting the most sensitive species, so only the sensitive species for the corresponding variables were considered in the analysis. The strength of the linear association between each response variable and the O_3 indices was compared through Pearson correlation coefficients. The corresponding linear regressions with AOT40 and POD_y indices were enforced with an intercept of 100% when the linear fit differed by >5% of that value. Statistical analyses were developed using SPSS 14.0 (SPSS Inc.).

2.5. Exposure- and dose-based O_3 critical levels

Exposure and dose-based critical levels, CLe_c and CLe_f, for AOT40 and POD_y respectively were calculated using exposure and dose-response functions. Only statistically significant ($p < 0.05$) functions were considered appropriate for deriving CLe. Different CLe were calculated considering three percentages of effect: 10%, 15% and 20%. The uncertainty ranges for the CLe were obtained using 95% confidence intervals of the regression lines according to the methodology described in González-Fernandez et al. (2014). Linear regressions were performed using the SPSS 14.0 (SPSS Inc.) statistical package.

3. Results

3.1. Linear correlation between effects and ozone indices

The sensitivity to O_3 of the annual species differed depending on the parameters analysed and the O_3 index considered. Table 2 shows the direction, robustness and significance of the correlations between parameters and indices, though the amount of information available for each parameter is different. The number of data (n) included in the analyses is indicated in Table 2. In general, correlation coefficients indicate the existence of a linear relationship for all parameters and the different O_3 indices, from moderate to very high, and ranged from $r = 0.38$ to $r = -0.96$, but with significance levels mostly $p < 0.001$. All the biomass parameters presented negative correlations, except for senescent biomass.

Considering the different POD_y flux thresholds tested, the POD6 is the index that best correlates with all biomass parameters, increasing by an average of 3% when the flux threshold increases from 0 to 6 nmol m^{-2} s^{-1}. Therefore, the results described more extensively below are centred in the relationship between POD6 and the biomass parameters. The total aerial and root biomass, and the total biomass, present a very good correlation with this flux index, with respective Pearson coefficients of −0.80, −0.79 and −0.78 ($p < 0.001$ for all three). The senescent biomass is the variable that presented the most consistent response to O_3: ten species showed increases in senescent biomass with increasing O_3 exposure. Thus, the amount of information available to analyse its correlation with the different O_3 indices is also the highest. However the results show a greater dispersion resulting in a lower r value ($r = 0.44$), although still maintaining the same level of significance ($p < 0.001$). The green biomass presents an intermediate r value compared with the other biomass variables ($r = 0.65$, $p < 0.001$). Concerning the two calculated ratios, senescent/green and root/above-ground biomass, the correlations with flux indices are also slightly improved when the flux threshold increases, with the POD6 the best fitting index. The directions of the relationship for the two ratios are in opposite direction: positive for the senescent/green ratio due to the greater effect of O_3 on senescent biomass compared with the green ($r = 0.54$, $p < 0.001$); and negative for the root/above-ground ratio due to the greater O_3-induced reduction on roots compared with the observed effects on the aerial biomass ($r = -0.72$, $p < 0.001$).

Coinciding with the findings for POD6, total above-ground, root and total biomass are the biomass parameters that best correlate with the AOT40 index: r values are respectively −0.70, −0.83 and −0.79 ($p < 0.001$ for all three). The green biomass has an $r = -0.66$ ($p < 0.001$), while the r is lower for the senescent biomass ($r = 0.38$, $p < 0.001$). When the biomass ratios are considered, the relationships with AOT40 and POD6 indices are similar.

Considering the nutritional quality parameters, ADF is the only one that does not show a significant correlation with O_3. NDF and ADL are positively correlated with the pollutant, but the Pearson coefficient changes depending on the index considered. Decreasing flux thresholds from 6 to 0 nmol m^{-2} s^{-1} slightly improve the correlation for NDF, being maximum for POD0 ($r = 0.70$, $p < 0.05$); whereas the reverse is true for lignin with $r = 0.85$ for POD6 ($p < 0.001$). Correlations between NDF and the concentration-based index AOT40 are also high ($r = 0.63$, $p < 0.05$), whereas for lignin this index is not significant. In regards to the two nutritional quality indices, RFV and CFV, they are negatively

Table 2

Pearson correlation coefficients. NDF = neutral detergent fiber; ADF = acid detergent fiber; RFV = relative food value; CFV = consumable food value. Bold variables are the selected for defining CLe.

	POD0	POD1	POD3	POD6	AOT40	n
Green biomass	-0.58^{***}	-0.59^{***}	-0.62^{***}	-0.65^{***}	-0.66^{***}	30
Senescent biomass	$+0.40^{***}$	$+0.41^{***}$	$+0.42^{***}$	$+0.44^{***}$	$+0.38^{***}$	89
Senescent/green biomass rate	$+0.51^{***}$	$+0.52^{***}$	$+0.54^{***}$	$+0.55^{***}$	$+0.54^{***}$	89
Total above-ground biomass	$\mathbf{-0.77}^{***}$	$\mathbf{-0.78}^{***}$	$\mathbf{-0.79}^{***}$	$\mathbf{-0.80}^{***}$	$\mathbf{-0.70}^{***}$	**51**
Root biomass	-0.75^{***}	-0.76^{***}	-0.77^{***}	-0.79^{***}	-0.83^{***}	39
Total biomass	-0.75^{***}	-0.76^{***}	-0.77^{***}	-0.78^{***}	-0.79^{***}	39
Root/above-ground biomass rate	-0.70^{***}	-0.70^{***}	-0.71^{***}	-0.71^{***}	-0.72^{***}	30
NDF	$+0.70^{*}$	$+0.69^{*}$	$+0.67^{*}$	$+0.63^{*}$	$+0.63^{*}$	12
ADF	$+0.51$	$+0.50$	$+0.47$	$+0.42$	$+0.56$	12
Lignin	$+0.82^{***}$	$+0.82^{***}$	$+0.84^{***}$	$+0.85^{***}$	$+0.51$	15
RFV	-0.66^{*}	-0.64^{*}	-0.61^{*}	-0.56	-0.64^{*}	12
CFV	$\mathbf{-0.96}^{***}$	$\mathbf{-0.96}^{***}$	$\mathbf{-0.96}^{***}$	$\mathbf{-0.96}^{***}$	$\mathbf{-0.79}^{*}$	**9**
Reproductive capacity (45 days)	$\mathbf{-0.82}^{***}$	$\mathbf{-0.83}^{***}$	$\mathbf{-0.84}^{***}$	$\mathbf{-0.84}^{***}$	$\mathbf{-0.76}^{***}$	**15**
Reproductive capacity (end)	-0.70^{**}	-0.73^{**}	-0.78^{***}	-0.82^{***}	-0.75^{**}	15

Significance level Pearson correlation coefficient: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

correlated with O_3. The best correlation found for RFV is with the flux-based index POD0 ($r = -0.66$, $p < 0.05$) and with the concentration based index AOT40 ($r = -0.64$, $p < 0.05$). CFV presents the highest negative correlations found in the present study ($r = -0.96$, $p < 0.001$) and no changes are observed for the different flux thresholds considered. The relationships of CFV and AOT40, though lower compared with the POD_y indices, is still significantly high ($r = -0.79$, $p < 0.05$).

The reproductive capacity, based on seed and flower production, is better correlated with O_3 when the 45 days-accumulation period is considered (from the start of the O_3 exposure until harvesting) compared with the 71 days-accumulated period (from the start of exposure till final seed maturation) for both AOT40 and POD_y indices. The best correlations are found considering POD3 and POD6 indices ($r = -0.84$, $p < 0.001$), although the differences among the different flux thresholds are small (the lowest is POD0 with an $r = -0.82$, $p < 0.001$). The correlation between the O_3 effects on the reproductive parameters is better considering POD_y-based indices than the concentration-based index AOT40 ($r = -0.76$, $p < 0.001$).

3.2. Selection of O_3-response variables and O_3 indices for the calculation of critical levels

Three variables were selected for CLe calculation: the total above-ground biomass (biomass for forage), CFV (nutritional quality of the forage) and seeds/flowers production (reproductive capacity and biodiversity).

Small differences were found among the Pearson correlation coefficients of the selected response parameters for the different POD flux thresholds considered, ranging by 0.03 and 0.02 for biomass and reproductive capacity respectively and showing no difference at all for the CFV (Table 2). Hence, the POD1 was selected for the definition of the CLe_f, in agreement with the current POD1-based O_3 CLe set for herbaceous vegetation dominated by perennial species within the CLRTAP (2010). AOT40 was also selected for the definition of the CLe_c, following the criteria of the CLRTAP.

3.3. Ozone exposure- and dose-response functions

Figs. 1 and 2 presents the exposure-(AOT40) and dose-response (POD1) functions for above-ground biomass, CFV and reproductive capacity. Equations, confidence intervals of the regression parameters, R^2 and p-value are also indicated in Table 3. Although all functions were statistically significant ($p < 0.001$), differences based on the coefficients of determination can be found depending on the variable considered and the O_3 index. The R^2 coefficients for AOT40 were 0.41 ($n = 51$) for above-ground biomass, 0.57 ($n = 9$) for CFV and 0.45 ($n = 15$) for flower and seed biomass. The coefficients of determination were higher for all three variables in the dose-response functions with POD1: 0.60, 0.69 and 0.90 for biomass, reproductive capacity and CFV respectively.

The exposure-response functions (Table 3) showed the steepest slope for the reproductive capacity followed by CFV and the above-ground biomass, although the 95% confidence intervals presented a high degree of overlap between the different functions. However, considering the dose-response functions the steepest slope was for CFV, followed by the reproductive capacity and the above-ground biomass. In this case the CFV and reproductive capacity presented some overlapping, well differentiated from the slope for total above-ground biomass.

3.4. Ozone critical levels

Table 4 presents the CLe_c and CLe_f calculated from the exposure and dose-response relationships, based on AOT40 and POD1 indices respectively, and their corresponding confidence intervals that provide information on the quality of the datasets and the robustness of the CLe.

Different CLe_c and different confidence intervals have been obtained considering an effect of 10%, 15% or 20% compared with the CFA treatment. In agreement with the slopes of the exposure-response functions, the reproductive capacity has the lowest CLe_c followed by CFV and the above-ground biomass, with AOT40 values ranging from 1998 to

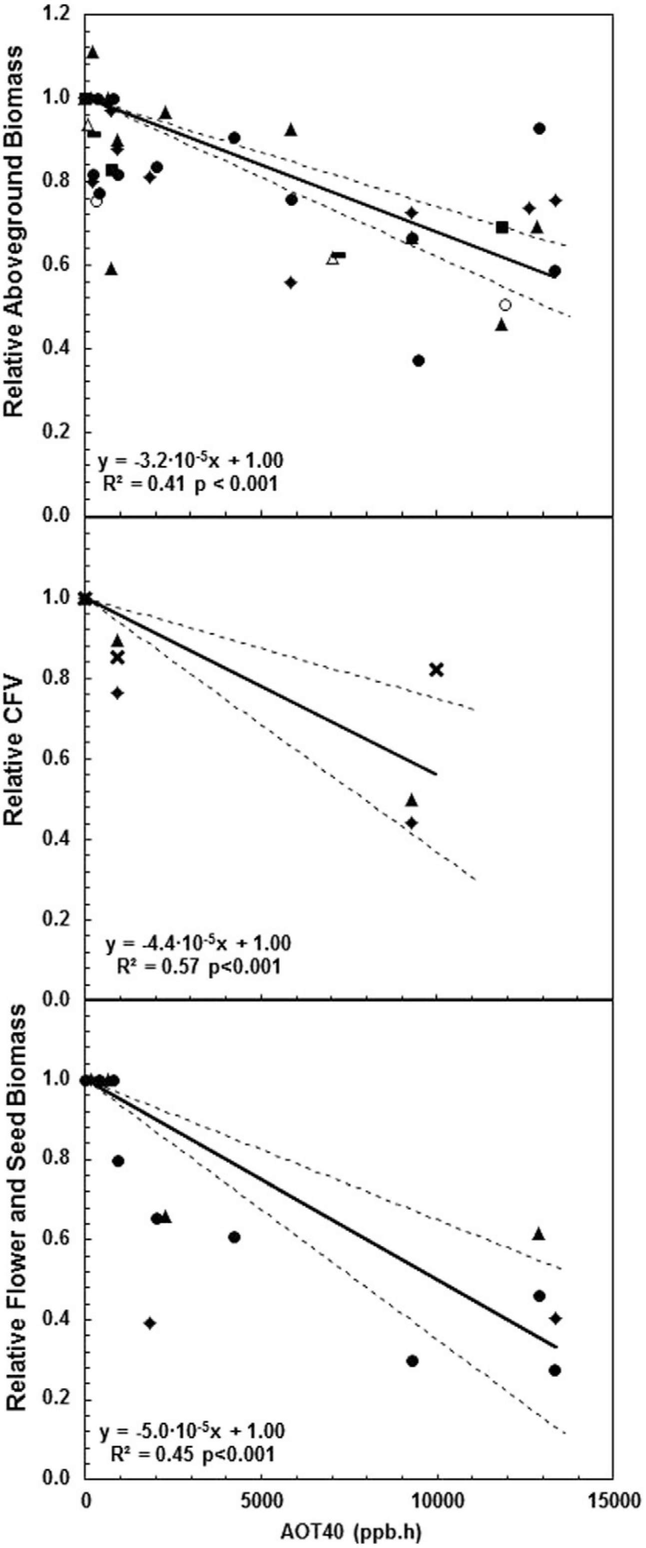

Fig. 1. AOT40-based exposure-response relationships for annual Mediterranean Dehesa pasture species based on O_3 effects on aboveground biomass, consumable food value (CFV) and seed and flower biomass. Dotted lines depict the 95% confidence intervals of the regression line. Symbols represent different species included in each relationship: ● *Trifolium striatum*; ▲*Trifolium cherleri*; ♦ *Trifolium subterraneum*; ■ *Trifolium glomeratum*; ▬ *Trifolium angustifolium*; ○ *Biserrula pelecinus*; △ *Medicago minima*; x *Cynosurus echinatus*.

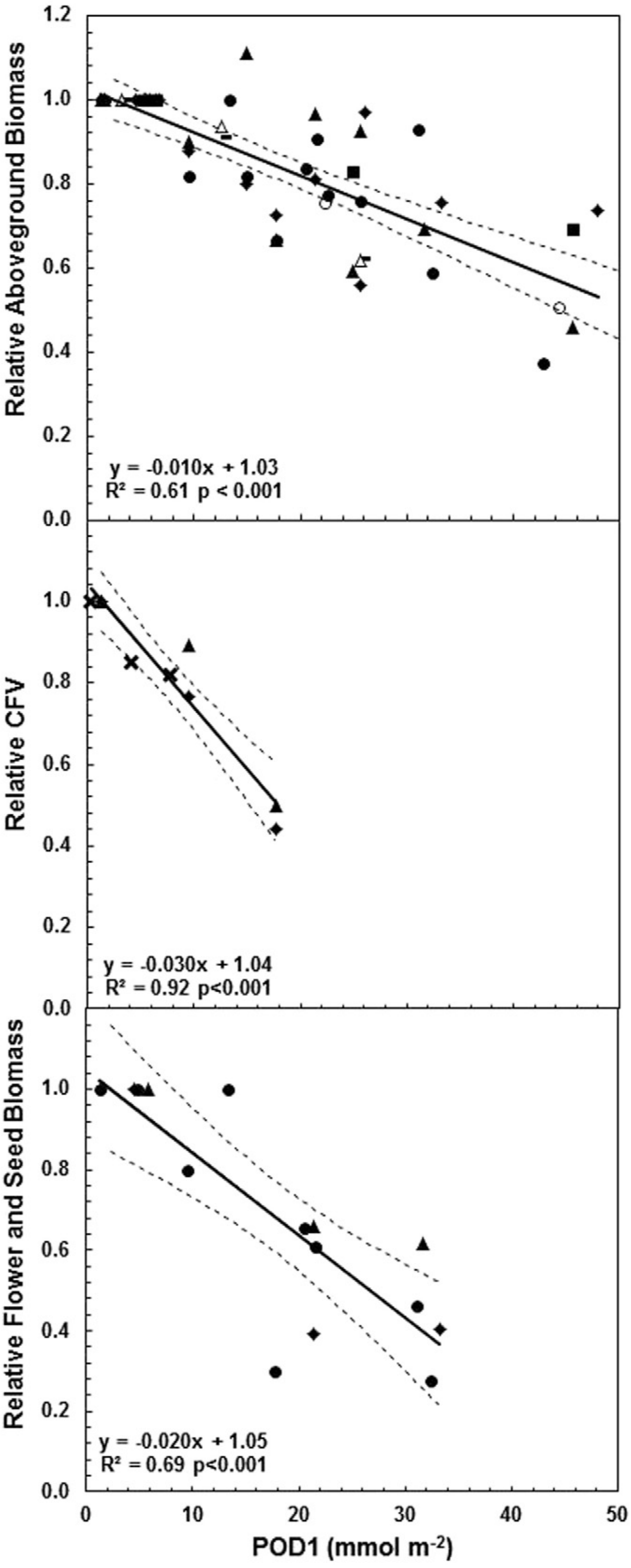

Fig. 2. POD1-based dose-response relationships for annual Mediterranean Dehesa pasture species based on O$_3$ effects on aboveground biomass, consumable food value (CFV) and seed and flower biomass. Dotted lines depict the 95% confidence intervals of the regression line. Symbols represent different species included in each relationship: ● *Trifolium striatum*; ▲*Trifolium cherleri*; ♦ *Trifolium subterraneum*; ■ *Trifolium glomeratum*; ▬ *Trifolium angustifolium*; ○ *Biserrula pelecinus*; △ *Medicago minima*; x *Cynosurus echinatus*.

3116 ppb h for a 10% of effect. The number and the dispersion of the data along the Y axis included for building the response functions and its adjustment affects the estimation of each CLe$_c$ uncertainty thresholds. The range between the lower and upper limit of the confidence interval was 39% for the above-ground CLe$_c$ while it increased up to 66% for the reproductive capacity, and up to 106% for the CFV for the same threshold effect of 10%. The uncertainty thresholds for the three CLe$_c$ show a considerable degree of overlapping (Table 4).

The CLe$_f$ based on POD1 pointed out to the CFV as the most sensitive parameter, followed by the reproductive capacity and finally the above-ground biomass. The CLe$_f$ for CFV and the reproductive capacity were in the same range, 4.6 and 7.2 mmol m^{-2} respectively for a 10% effect, but considerably lower than that for the above-ground biomass (12.2 mmol m^{-2}). The uncertainty thresholds for CLe$_f$ also changed depending on the parameter considered: the range of the CLe$_f$ confidence interval (10% effect) was 54% for above-ground biomass, followed by CFV with 83%, and the reproductive capacity with 169%. Considering all the calculated CLe$_f$ and their confidence intervals, CLe$_f$ for CFV were always lower than for the above-ground biomass (Table 4). However, the confidence intervals for the reproductive capacity overlap with those of the CFV and the above-ground biomass, except for an effect of 20%.

The CLe$_f$ increase linearly with the intensity of the estimated effect but the confidence intervals do not show the same trend (Table 4). In the case of the above-ground biomass CLe$_f$ the narrowest confidence interval is found for a 15% biomass reduction while it is increased if a 10% or a 20% effect is selected. The CFV and the reproductive capacity CLe$_f$ become more uncertain as the % effect decreases from 20 to 10% since the confidence intervals are the narrowest for a 20% effect.

4. Discussion

The present study analyses all available datasets regarding O$_3$ effects on therophytic species characteristic of Mediterranean Dehesa pastures based on OTC experiments and plants grown in pots. While the criteria for establishing CLes for tree species and crops are relatively clear, the derivation of CLes for herbaceous semi-natural vegetation has presented greater difficulties and it still has high uncertainties (CLRTAP, 2010). This is due to the diversity of the herbaceous communities and the number of species constituting these ecosystems, with complex interactions between them, environmental factors and management (Caballero et al., 2009). Therefore, the present study represents an important contribution with regard to the quantification of the risks of damage from tropospheric O$_3$ to European grasslands.

The review of the results of the experiments shows that O$_3$ is able to induce negative effects on yield, nutritional quality and reproductive capacity of the annual species and therefore it has the potential to impact on ecological processes, structure and biodiversity of this type of valuable grassland communities. The response variables selected to define the CLes attempt to describe the main effects of O$_3$ on the functioning of these ecosystems.

4.1. Critical levels

The CLes are by definition designed to protect the most sensitive species (CLRTAP, 2010), hence the development of the exposure and dose-response functions has considered only the sensitive species for each of the parameters assessed. Besides, a 10% of effect with regards CFA treatment has been considered in the CLes definition, in agreement with previously established CLes for perennial herbaceous species (CLRTAP, 2010). The scientific consensus establishes that the definition of CLes should be based on the greatest number of experiments conducted in different years. Moreover, when appropriate, experiments covering different climatic zones of the natural distribution of the species or communities assessed should also be included, so that the

Table 3
Exposure (AOT40) and dose (POD1)-response relationships for calculating critical levels. CFV = consumable food value.

	N of tests	N of points	Families (sps number)	Equation	Confidence interval	R^2	p
AOT40							
Above-ground biomass	5	51	Leguminosae (7)	$-0.0032\times + 100^*$	Slope: $(-0.0038, -0.0026)$	0.41	<0.001
CFV	1	9	Leguminosae (2) Gramineae (1)	$-0.0044\times + 100^*$	Slope: $(-0.0063, -0.0025)$	0.57	<0.001
Reproductive capacity	3	15	Leguminosae (3)	$-0.0050\times + 100^*$	Slope: $(-0.0065, -0.0035)$	0.45	<0.001
POD1							
Above-ground biomass	5	51	Leguminosae (7)	$-1.0276 + 102.51$	(97.25, 107.77) $(-1.26, -0.079)$	0.60	<0.001
CFV	1	9	Leguminosae (2) Gramineae (1)	$-3.004 + 103.8$	(95.77, 111.89) $(-3.82, -2.19)$	0.90	<0.001
Reproductive capacity	3	15	Leguminosae (3)	$-2.0506 + 104.8$	(87.5, 104.8) $(-2.88, -2.05)$	0.69	<0.001

* The ordinate at the intercept adjusted to 100.

variability caused by different environmental conditions that may modulate the responses of the plants to O_3 are collected where possible.

The selection of the response variable is also a key process for the definition of these thresholds in order to quantify properly the damage that the pollutant can cause on ecosystem services. Three parameters have been selected for calculating CLes for Mediterranean annual Dehesa-type grasslands based on the available information covering the widest range possible of species and climatic conditions, its relevance to ecosystem services provided by the annual pastures and the statistical significance of the linear relationship between O_3 indices and the variables analysed: the total above-ground biomass, the consumable food value (CFV) and the production of seeds and flowers referred to as the reproductive capacity.

The above-ground biomass is a parameter easy to evaluate and it provides rapid and definitive information about the vegetation forage yield, an economically important ecosystem service of pastures. This variable has also been chosen to describe the effects of O_3 on other types of herbaceous (semi- natural) vegetation in the context of the CLRTAP, hence enabling O_3 risk damage comparisons between different grassland communities. The CLes for above-ground biomass production presented in this study have been derived from 5 experiments including 8 species; and therefore, they can be considered as the most robust CLes of all the proposed.

Ozone effects on above-ground and root biomass can have an important influence on the biogeochemical cycles of water, nitrogen and carbon and they have been related to the potential of semi-natural ecosystems to sequester carbon (C) in soils (CLRTAP, 2010). Carbon from dry roots and above-ground biomass left at the end of the growing cycle of the annual pastures is mainly stored in the first few centimetres of the soil (soil organic carbon SOC). Although the ability of the Dehesa soils for sequestering C is low compared with other Mediterranean ecosystems, its potential could be altered by the O_3 effects on biomass production (Rodeghiero et al., 2011). However, the actual assessment of the O_3 effects on C sequestration for annual dehesa communities cannot be entirely based on the effects on biomass, which is only one of its indicators. For instance, the results obtained regarding the lignin content of the foliage, which increased under O_3 exposure for the five species tested, may also imply an effect on C cycling. A higher concentration of lignin can slow down the pasture biomass decomposition, increasing the residence time of organic matter on the ground and affecting the soil C storage processes (Booker et al., 2005). Thus, other factors besides biomass would need to be considered for the assessment of O_3 effects on the C sequestration potential of annual Mediterranean Dehesa pastures.

The CFV index integrates biomass production and digestibility for cattle, giving information about both yield and nutritional quality of the forage (Krupa et al., 2004). The exposure and dose-response functions for the CFV presented a strong and significant linearity with steeper slopes than the above-ground biomass, indicating a greater O_3-sensitivity. The effects on the CFV and other forage quality related parameters can be related to provisioning ecosystem services such as food production. The effects of O_3 on the forage quality of semi-natural grassland species have been described by several studies (e.g. Bender et al., 2006; Muntifering et al., 2006; González-Fernández et al., 2008) and its potential impact on live weight gain of livestock grazing on semi-natural communities has been explored by Hayes et al. (2016). Hayes et al. (2016) show, across a range of grazed semi-natural vegetation communities of the UK, how increasing O_3 concentrations may be able to reduce the production of livestock unless supplementary feeding is provided to compensate for these losses. Thus, the CFV and other pasture quality variables provide information on the effects of O_3 on the quality of pastures as a food resource for herbivorous animals that the effects on above-ground biomass cannot provide. However, in this analysis the response functions derived for the CFV index are based on a single experiment with three species. Thus, the current available information is not considered sufficient for providing reliable CLes and they should be considered as provisional until more information becomes available.

The effect of O_3 on the reproductive capacity of the pasture has been described by the relationship between the O_3 exposure or dose and the seed and flower production. Seed production is a key parameter directly related with the long term biodiversity of annual herbaceous communities that develop annually from the soil seed bank (Peco et al., 2009).

Table 4
Ozone exposure (AOT40) and flux (POD1)-based critical levels. Intervals of Confidence between brackets. CFV = Consumable Food Value.

	Effect threshold		
	10%	15%	20%
Total above-ground biomass			
AOT40 (ppb h)	3116 (2631, 3846)	4674 (3947, 5769)	6232 (5263, 7692)
POD1 (mmol m^{-2})	12.18 (8.88, 15.47)	17.04 (14.02, 20.06)	21.91 (18.75, 25.06)
CFV			
AOT40 (ppb h)	2272 (1587, 4000)	3407 (2380, 6000)	4543 (3174, 8000)
POD1 (mmol m^{-2})	4.60 (2.71, 6.50)	6.27 (4.52, 8.01)	7.93 (6.23, 9.64)
Reproductive capacity			
AOT40 (ppb h)	1998 (1538, 2857)	2997 (2308, 4286)	3996 (3076, 5714)
POD1 (mmol m^{-2})	7.22 (1.10, 13.34)	9.66 (4.20, 15.12)	12.10 (7.17, 17.02)

The negative effect of O_3 on the seed production of legume species described in this study may be of particular importance for the ecosystem functioning. A lower proportion of this family in the pasture composition causes a decrease in the nutritional value of the forage for herbivores due to the contribution of this family to the pasture protein content (Olea and San Miguel-Ayanz, 2006). Moreover, a reduction of the legume fraction has direct consequences on decreasing atmospheric nitrogen fixation (Hewitt et al., 2014). The CLes obtained for the reproductive capacity are based on exposure and dose-response functions stepper than the above-ground biomass functions, which resulted in lower CLes for this parameter. The data available, 3 independent experiments and 3 species, was considered enough for setting an O_3 critical level. However, the exposure and dose-response relationships would benefit from future experimental work that widens the empirical basis for this CLe.

The results presented in this study expand the scientific knowledge supporting the European CLes for European semi- natural vegetation, providing new information for an important Mediterranean pasture community. The CLRTAP considers different CLes for the assessment of the risk of O_3 damage on herbaceous vegetation (CLRTAP, 2010). The initial CLes proposal and subsequent revisions were based on experiments considering mostly perennial species; only a single experiment with annual species from Mediterranean Dehesa communities was incorporated more recently (CLRTAP, 2010). Based on this information, a provisional CLe_c to protect annual sensitive species was set at 3 ppm h accumulated over a period of 3-months or the growing season, and considering a 10% loss of the above-ground and seed production (CLRTAP, 2010). The present study develops for the first time the exposure-response function supporting this value for biomass loss, although a 45-day period of accumulation is indicated for matching with the mean length of the O_3 exposure period for the OTC studies in which the functions are based on. However, a second set of CLe_c for reproductive capacity shows that O_3 effects may be happening below the CLe for above-ground biomass and thus this parameter should be used to protect these plant communities from O_3 injury. Moreover, the new CLe_f, set up at 12.2 and 7.2 mmol m^{-2} accumulated over 45 days for a 10% loss of the above-ground biomass and reproductive capacity respectively, will allow more precise O_3 risk assessments because they consider the modulation played by environmental growing conditions on O_3 sensitivity. The CLe_f for annual dehesa pasture species contrasts with the CLe_f set for productive perennial grasslands and for high conservation value grasslands at POD1 $= 2$ mmol m^{-2} accumulated over 3 months for a 10% loss in the above-ground biomass (CLRTAP, 2010). However, the comparison between the CLe_f is not straightforward because they are affected by the species-specific parameterizations of the DO_3SE model used to estimate the O_3 stomatal flux. The comparison of the DO_3SE parameterizations for perennial and annual *Trifolium* species shows that the g_{max} of the annuals are higher, which is in accordance with their high growth rates during the spring and explains the large O_3 fluxes accumulated in relatively short periods.

The present study includes also provisional CLe_c and CLe_f, for the CFV index that integrates yield and nutritional quality. It is essential for reaching the level of robustness required to be considered as definitive CLes to make a major effort in developing experimental studies about O_3 effects on grasslands considering forage quality parameters. The new thresholds highlight the conservative character of the CLes set for above-ground biomass, as some O_3 effects might occur below these thresholds.

4.2. Calculating critical level exceedances for risk assessments

Phenological dynamics of Dehesa annual communities follow a pattern characteristic of the Mediterranean climate linked to the availability of soil water. The biomass growth is linked to humid periods (from early autumn until spring) while the seed form is adopted during the characteristic dry summers. Through autumn, when rains return after several months of drought, seeds from the soil bank germinate, but new seedlings remain dormant due to the low winter temperatures. A quick growth resumes with the increment of the spring temperatures closely followed by the reproductive season which concludes with seed maturity and plant death.

The duration of the growing cycle of the pasture and the timing for plant death depends on the amount and distribution of rainfall, characterized by its marked yearly irregularity (Peco et al., 2009). Therefore, the length and end of the growth season can suffer strong inter-annual variations also modulated by the local conditions, complicating the risk assessment studies due to the difficulty in defining the exposure period (González-Fernández et al., 2010).

Aiming to cope with this variability, it is recommended as a general rule for the calculation of CLes exceedances, that the 45-days accumulation period fit within the general spring growing season of the Dehesa climatic types 1 and 2, which are the most extensively distributed in Spain (82% of the Spanish Dehesas included) (Roig et al., 2013) starting on DOY $= 30$ (1st February) and finishing on DOY $= 151$ (31th May). However, a wider period starting on DOY $= 60$ (1st March) until DOY $= 181$ (30th June) can be considered for the climatic type 3 Dehesas (18% of the Spanish Dehesas included) and more local scale risk assessment exercises. This wider spring growing period is supported by a 2-year field study that showed the active physiology and growth of annual pastures which constitute the understory of a Dehesa type 3 (González-Fernández et al., 2010). A 45-day running accumulation period within the spring growing periods described above is suggested for calculating O_3 CLe_c and CLe_f exceedances.

4.3. Mapping critical levels of annual Mediterranean pastures

The O_3 CLe established for the CLRTAP constitute environmental policy tools for mapping, at continental scale, the potential risk of O_3 damage on European vegetation. The CLRTAP Mapping Manual (CLRTAP, 2010) suggests that mapping the CLe exceedances for semi-natural vegetation communities may be based on the habitat classification of the European Nature Information System (EUNIS) (EEA, 2015) but does not identify the EUNIS categories considered by the CLe already present in the Manual.

The CLes presented in this study should be applied to the EUNIS "E7.3 class (Dehesa), sparsely wooded grasslands (canopy cover 5–10% trees) characteristic of Mediterranean type climate". Dehesas are mainly distributed in the centre and south-west of the Iberian Peninsula. Their edaphoclimatic conditions and different managements allow a collage of annual grasslands (therophytic pastures) and annual/perennial mixed grasslands (xero-mesophytic pastures). These general grassland types include different annual communities suitable for the new CLes application: acidophilic therophytic pastures on non-sandy soils (Helianthemetalia), therophytic sandy pastures (Malcomietalia) and post-tillage therophytic pastures (Thero-Brometalia). These communities are not exclusive of the Dehesa habitat, but also belong to other EUNIS categories: E1.3, Mediterranean xeric grassland; E1.6, subnitrophilous annual grassland; and E1.8, closed Mediterranean dry acid and neutral grassland. Therefore, the CLes proposed in this study could also be applied to these EUNIS categories until more specific information about the O_3 response of these communities becomes available.

4.4. Limitations and recommendations

The new CLes for annual pastures presented in this study, like other CLe currently available for different vegetation types (CLRTAP, 2010), are based on OTCs experiments. The use of OTC can imply some differences in the O_3 absorption fluxes compared with plants growing in the field. The use of OTCs can increase the O_3 flux due to the effect of the forced ventilation of the fumigation systems (Nussbaum and Fuhrer, 2000). However, the use of the OTC system has also some advantages by allowing a precise definition of the O_3 treatment levels above and below current ambient concentrations. Having an adequate control treatment reproducing background O_3 concentrations is essential in the calculation of the relative losses caused by the levels of the pollutant.

The experiments considered in this combined analysis used plants growing in pots individually or in simple mixtures with an O_3 tolerant competitor species. However, the annual Dehesa pastures constitute species-rich communities where some species have been described as O_3-tolerant (Gimeno et al., 2004a). The addition of the O_3 factor in a complex community with limited resources might change the competition relationships among species which can in return affect the response of the community to O_3. Calvete-Sogo et al. (2014) conducted an O_3 fumigation experiment in OTCs with direct seeding on the ground of a mixture of six annual species (three families) with different sensitivities to O_3. The observed effects on the above-ground biomass of the whole canopy under similar AOT40 exposures are consistent with those from the pot experiments of the present analysis, although the effects were less intense. Other O_3 fumigation experiments with complex species mixtures or intact communities have also shown lower responses to O_3 than individual species (Bassin et al., 2007a). Several reasons have been proposed to explain this difference like the functional redundancy of the species within the community, the genetic variability in O_3 sensitivity and the competition interactions between the species (Bassin et al., 2007b). The presence of O_3-tolerant species in the community in the Calvete-Sogo et al. (2014) experiment helped to reduce the O_3-effect on the above-ground biomass at canopy scale. However, other factors may have contributed to this difference. The competition for the limited supply of water, nutrients and space in the Calvete-Sogo et al. (2014) experiment, in contrast with the plentiful supply of water and, for some experiments, nutrients in the potted plants may have also contributed to diminish the negative effect of the pollutant. These factors can limit the growth of the plants, which in fact is lower compared with potted grown plants, thus reducing the O_3 absorbed dose and potentially the effect of O_3.

The quantification of the O_3 effects on complex grassland ecosystems should be based on the responses found at the community scale. However, it is interesting to note that the interpretation of the results obtained from intact or field grown communities are also subjected to many uncontrolled spatial and temporal factors which complicate the analysis of the O_3 effect (Stampfli and Fuhrer, 2010). The studies based on individual species or simple mixtures can provide valuable information for understanding the results obtained from community scale experiments. However, the effects identified at the species levels should only be interpreted as a risk of an effect for the whole community. Furthermore, the new CLes for Mediterranean annuals are based on the most sensitive species responding to each of the parameters assessed. Thus, if these CLe are used to quantify the effects of O_3 on Mediterranean pasture communities, an overestimation of the damage would be expected.

The potential interactions of other factors like nitrogen availability, known to interact with the O_3 response of these annual pasture species (Sanz et al., 2005, 2007, 2011, 2013, 2014), was not directly considered in the exposure- and dose-response relationships derived in this study. This is a common characteristic of current O_3 CLe that limits their applicability for quantifying O_3 induced effects under field conditions, where these interactive factors may vary at the local scale. However, in this study, the average response to O_3 across interactive experimental treatments (Table 1) was used to derive the response functions so the relationships can be interpreted as the average response to O_3 under different nitrogen availability and inter-specific competition conditions.

The above mentioned limitations make the new CLes defined in the present study suitable for risk assessment analysis but not for quantifying damages. The uncertainties linked intrinsically with the CLes calculation regarding the variability of the species/populations sensitivity, the variability of the experimental conditions, the artefacts caused by the experimentation and the significance of the regression lines, making essential the consideration of confidence intervals of the CLes to indicate the robustness of the value. For improving O_3 risk assessments, future studies should focus on canopy scale responses that can cope with the complexity of the herbaceous communities. These new approaches should consider multi-species response and flux calculations in the dose-response functions from which CLe at canopy scale can be derived.

5. Conclusions

A new set of O_3 CLes with 95% confidence intervals, derived following the methodology of the CLRTAP, are proposed for semi-natural vegetation dominated by annual Mediterranean species. AOT40- and POD1-based CLe_c and CLe_f, accumulated over a 45 days period, are established (with confidence levels between brackets) for a 10% impact on the above-ground biomass and reproductive capacity at AOT40 = 3.1 (2.6, 3.8) ppm h and 2.0 (1.5, 2.8) ppm h and POD1 = 12.2 (8.9, 15.5) mmol m^{-2} and 7.2 (1.1, 13.3) mmol m^{-2} respectively. The AOT40-based CLe_c for above-ground biomass confirms the value already established by the CLRTAP (2010) but the CLes based on the reproductive capacity show that potential O_3 effects may be happening below this level. The POD1-based CLe_f are considered more suitable for describing the risk of O_3 effects.

Another set of CLe are also proposed for forage quality. The CLe for forage quality is based on the CFV index: AOT40 = 2.3 (1.6, 4.0) ppm h and POD1 = 4.6 (2.7, 6.5) mmol m^{-2}. All CLe are accumulated over 45 days. Despite being more sensitive to O_3 than biomass-based CLe, though less that the reproductive capacity CLe, it should be considered as provisional due to the limited amount of experimental data available.

These CLe contribute to improving the risk assessment of O_3 effects on European grassland ecosystems but are not suitable for quantifying O_3 effects because the response of tolerant species and the effects over the whole community are not taken into account. The proposed CLes are suitable for estimating the risk of O_3 damage in the EUNIS categories E7.3, E1.3, E1.6 and E1.8 which are dominated by annual Mediterranean species. It is recommended that the CLes are accompanied by the confidence intervals reflecting the variability of the exposure and dose-response functions and the robustness of the CLes.

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References

Alonso, R., Elvira, S., Castillo, F.J., Gimeno, B.S., 2001. Interactive effects of ozone and drought stress on pigments and activities of oxidative enzymes in *Pinus halepensis*. Plant Cell Environ. 24, 905–916.

Alonso, R., Bermejo, V., Sanz, J., Valls, B., Elvira, S., Gimeno, B.S., 2007. Stomatal conductance of semi-natural Mediterranean grasslands: implications for the development of critical levels. Environ. Pollut. 146, 692–698.

Bassin, S., Volk, M., Suter, M., Buchmann, N., Fuhrer, J., 2007a. Nitrogen deposition but not ozone affects productivity and community composition of subalpine grassland after 3 year of treatment. New Phytol. 175, 523–534.

Bassin, S., Volk, M., Fuhrer, J., 2007b. Factors affecting the ozone sensitivity of temperate European grasslands. An overview. Environ. Pollut. 146, 678–691.

Bender, J., Muntifering, R.B., Lin, J.C., Weigel, H.J., 2006. Growth and nutritive quality of *Poa pratensis* as influenced by ozone and competition. Environ. Pollut. 142 (1), 109–115.

Bermejo, V., Gimeno, B.S., Sanz, J., de la Torre, D., Gil, J.M., 2003. Assessment of the ozone sensitivity of 22 native plant species from Mediterranean annual pastures based on visible injury. Atmos. Environ. 37, 4667–4677.

Booker, F.L., Prior, S.A., Torbert, H.A., Fiscus, E.L., Pursley, W.A., Hus, S., 2005. Decomposition of soybean grown underelevated concentrationsof CO_2 and O_3. Glob. Chang. Biol. 11, 685–698.

Caballero, R., Fernández-González, F., Perez Badia, R., Molle, G., et al., 2009. Grazing systems and biodiversity in Mediterranean areas: Spain, Italy and Greece. Pastos 39, 9–152.

Calvete-Sogo, H., Elvira, S., Sanz, J., González-Fernández, I., García-Gómez, H., Sánchez-Martín, L., Alonso, R., Bermejo, V., 2014. Current ozone levels threaten gross primary production and yield of Mediterranean annual pastures and nitrogen modulates the response. Atmos. Environ. 95, 197–206.

Calvete-Sogo, H., González-Fernández, I., Sanz, J., Elvira, S., Alonso, R., García-Gómez, H., Ibáñez-Ruiz, M.A., Bermejo-Bermejo, V., 2016. Heterogeneous responses of component species to ozone and nitrogen alter the species composition of Mediterranean annual pastures. Oecologia http://dx.doi.org/10.1007/s00442-016-3628-z.

Chaudhary, N., Agrawal, S.B., 2013. Intraspecific responses of six Indian clover cultivars under ambient and elevated levels of ozone. Environ. Sci. Pollut. Res. 20, 5318–5329.

CLRTAP, 2010. Mapping critical levels for vegetation, chapter III of manual on methodologies and criteria for modelling and mapping critical loads and levels and air pollution effects, risks and trends. UNECE convention on long-range Transboundary air pollution (accessed on [date of consultation] on Web at www.icpmapping.org).

EEA, 2015. web site: http://eunis.eea.eu.int/index.jsp.

García del Barrio, J.M., Alonso, R., Benavides, R., Roig, S., 2014. Species richness and similarity of vascular plants in the Spanish dehesas at two spatial scales. For. Syst. 23 (1), 111–119.

Gimeno, B.S., Bermejo, V., Sanz, J., de la Torre, D., Elvira, S., 2004a. Growth response to ozone of annual species from Mediterranean pastures. Environ. Pollut. 132, 297–306.

Gimeno, B.S., Bermejo, V., Sanz, J., de la Torre, D., Gil, J.M., 2004b. Assessment of the effects of ozone exposure and plant competition on the reproductive ability of three therophytic clover species from Iberian pastures. Atmos. Environ. 38, 2295–2303.

González-Fernández, I., Bass, D., Muntifering, R., Mills, G., Barnes, J., 2008. Impacts of ozone pollution on productivity and forage quality of grass/clover swards. Atmos. Environ. 42, 8755–8769.

González-Fernández, I., Bermejo, V., Alonso, R., Elvira, S., Sanz, J., Gimeno, B.S., 2010. Modelling dehesa annual pasture dynamics: application to stomatal ozone deposition. Atmos. Environ. 44, 2507–2517.

González-Fernandez, I., Calvo, E., Gerosa, G., Bermejo, V., Marzuoli, R., Calatayud, V., Alonso, R., 2014. Setting ozone critical levels for protecting horticultural Mediterranean crops: case study of tomato. Environ. Pollut. 185, 178–187.

Hayes, F., Mills, G., Jones, L., Abbott, J., Ashmore, M., Barnes, J., Cape, J.N., Coyle, M., Peacock, S., Rintoul, N., Toet, S., Wedlich, K., Wyness, K., 2016. Consistent ozone-induced decreases in pasture forage quality across several grassland types and consequences for lamb production. Sci. Total Environ. 543 (A), 336–346.

Heggestad, H.E., Middleton, J.T., 1959. Ozone in high concentrations as a cause of tobacco leaf injury. Science 129, 208–210.

Hewitt, D.K.L., Mills, G., Hayes, F., Wilkinson, S., Davies, W., 2014. Highlighting the threat from current and near-future ozone pollution to clover in pasture. Environ. Pollut. 189, 111–117.

Jarvis, P.G., 1976. The interpretation of the variations in leaf water potential and stomatal conductance found in canopies in the field. Philos. Trans. R. Soc. Lond. B 273, 593–610.

Krupa, S., Muntifering, R., Chappelka, A., 2004. Effects of ozone on plant nutritive quality characteristics for ruminant animals. Botanica 54, 1–12.

Lenka, S., Lenka, N.K., 2012. Impact of tropospheric ozone on agroecosystem: an assessment. J. Agric. Phys. 12, 1–11.

Linn, J.G., Martin, N.P., 1989. Forage quality tests and interpretation. Ag-FO-02637. University of Minnesota Extension Service, Minneapolis.

Massman, W.J., 1998. A review of the molecular diffusivities of H_2O, CO_2, CH_4, CO, O_3, SO_2, NH_3, N_2O, NO, and NO_2 in air, O_2 and N_2 near STP. Atmos. Environ. 32, 1111–1127.

Marañon, T., 1985. Diversidad florística y heterogeneidad ambiental en una dehesa de Sierra Morena. An. Edafología Agrobiol. 44, 1183–1197.

Mills, G., Hayes, F., Simpson, D., et al., 2011a. Evidence of widespread effects of ozone on crops and (semi-)natural vegetation in Europe (1990–2006) in relation to AOT40- and fluxbased risk maps. Glob. Chang. Biol. 17, 592–613.

Mills, G., Pleijel, H., Braun, S., Büker, P., Bermejo, V., Calvo, E., Danielsson, H., Emberson, L., González-Fernández, I., Grünhage, L., Harmens, H., Hayes, F., Karlsson, P.E., Simpson, D., 2011b. New stomatal flux-based critical levels for ozone effects on vegetation. Atmos. Environ. 45, 5064–5068.

Muntifering, R.B., Chappelka, A.H., Lin, Karnosky, D.F., Somers, G.L., 2006. Chemical composition and digestibility of *Trifolium* exposed to elevated ozone and carbon dioxide in a free-air (FACE) fumigation system. Funct. Ecol. 20, 269–275.

Nussbaum, S., Fuhrer, J., 2000. Difference in ozone uptake in grassland species between open-top chambers and ambient air. Environ. Pollut. 109, 463–471.

Olea, L., San Miguel-Ayanz, A., 2006. The Spanish dehesa. A traditional Mediterranean silvopastoral system linking production and nature conservation. Grassl. Sci. Eur. 11, 1–15.

Peco, B., Rico, L., Azcárate, F.M., 2009. Seed size and response to rainfall patterns in annual grasslands: 16 years of permanent plots. J. Veg. Sci. 20, 8–16.

Richards, B.L., Middleton, J.T., Hewitt, W.B., 1958. Air pollution with relation to agronomic crops. V. Oxidant stipple of grape. Agron. J. 50, 559–561.

Rodeghiero, M., Rubio, A., ... La Mantia, T., 2011. Chapter 8: soil carbon in Mediterranean ecosystems and related management problems. In: Jandl, R., Rodeghiero, M., Olsson, M. (Eds.), Soil Carbon in Sensitive European Ecosystems: From Science to Land Management. Jhon Wiley & Sons. ISBN: 978-1-119-97001-9 (October 2011).

Roig, S., Evett, R., Gea-Izquierdo, G., Cañellas, I., Sánchez-Palomares, O., 2013. Climatic influence on oak landscape distributions. Mediterranean oak woodland working landscapes. Volume 16 of the Series Landscape Series, pp. 61–89 (Date:19 June 2013. ISBN:978–94–007-6706-5).

Sanz, J., Muntifering, R.B., Gimeno, B.S., Bermejo, V., Elvira, S., 2005. Ozone and increased nitrogen supply effects on the yield and nutritive quality of *Trifolium subterraneum*. Atmos. Environ. 39, 5899–5907.

Sanz, J., Bermejo, V., Gimeno, B.S., Elvira, S., Alonso, R., 2007. Ozone sensitivity of the Mediterranean terophyte *Trifolium striatum* is modulated by soil nitrogen content. Atmos. Environ. 41, 8952–8962.

Sanz, J., Bermejo, V., Muntifering, R., Gónzalez-Fernandez, I., Gimeno, B.S., Elvira, S., Alonso, R., 2011. Plant phenology, growth and nutritive quality of *Briza maxima*: responses induced by enhanced ozone atmospheric levels and nitrogen enrichment. Environ. Pollut. 159, 423–430.

Sanz, J., Calvete-Sogo, H., González-Fernández, I., Lin, J., García-Gómez, H., Muntifering, R., Alonso, R., Bermejo-Bermejo, V., 2013. Foliar senescence is the most sensitive response to ozone in *Bromus hordeaceus*, and is modulated by nitrogen input. Grass Forage Sci. http://dx.doi.org/10.1111/gfs.12090.

Sanz, J., González-Fernández, I., Calvete-Sogo, H., Lin, J.S., Alonso, R., Muntifering, R., Bermejo, V., 2014. Ozone and nitrogen effects on yield and nutritive quality of the annual legume *Trifolium cherleri*. Atmos. Environ. 94, 765–772.

Stampfli, A., Fuhrer, J., 2010. Spatial heterogeneity confounded ozone-exposure experiment in semi-natural grassland. Oecologia 162 (2), 515–522.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597.

Vazquez-de-Aldana, B.R., Garcia-Ciudad, A., Garcia-Criado, 2008. Interannual variations of aboveground biomass and nutritional quality of Mediterranean grasslands in Western Spain over a 20-year period. Aust. J. Agric. Res. 59, 769–779.

Weschler, C.J., 2006. Ozone's impact on public health: contributions from indoor exposures to ozone and products of ozone-initiated chemistry. Environ. Health Perspect. 114, 1489–1496.

Vingarzan, R., 2004. A review of surface ozone background levels and trends. Atmos. Environ. 38, 3431–3442.

4. DISCUSIÓN GENERAL

La dehesa es un ecosistema que se distribuye geográficamente en el centro y sur-oeste de la península Ibérica, con unas características edafoclimáticas y de gestión peculiares, que son responsables de un conjunto teselar de comunidades pascícolas de especies anuales (pastos terofíticos) y pascícolas mixtas de anuales y perennes (pastos xero-mesofíticos) (Olea & San Miguel, 2006). Estos pastos que forman parte del alimento de la fauna silvestre y la ganadería extensiva, presentan una gran variabilidad anual y estacional en cuanto a producción y calidad nutritiva, relacionada con el típico clima mediterráneo (Vázquez Aldana, 2008). Además, presentan una alta diversidad florística, llegándose a registrar 135 especies en 0.1 ha., siendo gramíneas, leguminosas y compuestas las familias con mayor representación (Marañón, 1985; García del Barrio).

En la actualidad, en zonas rurales extensas de la península Ibérica, donde la dehesa forma parte de su paisaje, es frecuente la presencia de niveles de O_3 potencialmente tóxicos para la vegetación (Notario et al., 2011; Fernández-Fernández, 2012, 2013), y de un depósito atmosférico de N en un rango de 10-38 kg ha^{-1} año (Sanz et al., 2002; Rodá et al., 2002; García-Gómez et al., 2014). Considerando tanto el depósito de O_3 como de N, el rango de valores registrados se encuentra por encima de los límites para asegurar la estructura y diversidad actual de este tipo de vegetación (Directiva de calidad de aire 2008/50/CE; CLRTAP, 2010; Bobbink et al., 2010).

Con el objetivo de evaluar el impacto del incremento de los niveles de O_3 troposférico en las comunidades herbáceas de la dehesa, analizar la modulación de la respuesta por la disponibilidad de N y proponer niveles críticos para este tipo de vegetación, siguiendo la metodología del CLRTAP, se desarrollaron diversos experimentos con especies terófitas de las familias *Leguminosae* (11 especies) y *Gramineae* (11 especies), con un elevado valor pascícola y ecológico. Todos ellos siguieron un diseño similar: las plantas crecieron en macetas sobre un sustrato artificial, se mantuvieron a capacidad de campo y el tiempo promedio de exposición al contaminante fue de 45 días. Todos los experimentos contaron con un tratamiento de aire filtrado (CFA) que se utilizó como control. En varios experimentos las plantas fueron expuestas a distintos tratamientos de N con el propósito de analizar la modulación que ejerce la disponibilidad de N en la respuesta al O_3.

Esta memoria presenta estos estudios pioneros realizados para evaluar los efectos del O_3 en especies herbáceas anuales de pastos mediterráneos. Hasta su inicio, toda la información disponible en la literatura científica se basaba en el análisis de efectos en herbáceas perennes. Gracias a la información experimental adquirida, se ha podido realizar en el capítulo 3 la primera aproximación a la definición de los niveles críticos de O_3 para estos tipos de comunidades.

Análisis de sensibilidad al ozono de especies pascícolas mediterráneas anuales

Los diferentes estreses ambientales a los que están sometidas las plantas, tanto de componente biótico como abiótico, pueden originar una sintomatología foliar específica válida como herramienta de detección del estrés (Vollenweider et al., 2005). Los primeros síntomas foliares visibles provocados por los niveles ambientales de O_3, se detectaron a finales de la década de los cincuenta en zonas agrarias de California (EEUU), (Richards et al., 1958; Heggestad and Middleton, 1959). Desde entonces se han encontrado daños foliares en muchas especies vegetales diseminadas en un gran número de países (Mills et al., 2011). Otro efecto en la vegetación, no específico, pero característico del O_3, es la aceleración de la senescencia foliar y por tanto, del incremento de biomasa senescente (Chaudhary & Agrawal, 2013).

Los resultados obtenidos en el primer experimento de la presente memoria, realizado para analizar la sensibilidad relativa al O_3 de 22 especies anuales en base a daños visibles específicos e incremento de senescencia (desarrollado en el subcapítulo 3.1), indicaron una sensibilidad elevada general de las especies analizadas, ya que el O_3 provocó el desarrollo de sintomatología visible en 16 de las 22 especies evaluadas. Sin embargo, aunque ambas familias, leguminosas y gramíneas, presentaron especies sensibles, hubo claras diferencias entre ambas relacionadas con el tipo de daño, el número de especies afectadas y la extensión de los daños.

Las especies pertenecientes a la familia de las leguminosas, especialmente las del género *Trifolium*, fueron más sensibles que las gramíneas, aunque también algunas leguminosas como *Ornithopus compressus*, *Anthyllis cornicina* y *Anthyllis lotoides* fueron tolerantes al contaminante. En la valoración de daños específicos visibles realizado, un 67% de las leguminosas mostraron sintomatología a niveles ambientales de O_3, con valores de AOT40 cercanos a 300 ppb h y bajo una exposición relativamente corta, mientras que tan sólo un 27 % de gramíneas mostraron sintomatología foliar y bajo una exposición mayor, con valores de AOT40 por encima de 1000 ppb h.

En concordancia con lo anterior, el análisis de la varianza de doble vía considerando familia y O_3 como factores en el análisis de hojas sanas, senescentes y con daños específicos, indica de forma clara un mayor impacto del O_3 en las leguminosas respecto a las gramíneas, a través de la interacción significativa entre los factores. Por ejemplo, mientras que la senescencia entre ambas familias es semejante en el tratamiento control (CFA), el incremento de senescencia con el O_3 puede llegar a ser un 37% más elevado en leguminosas que en gramíneas (tratamiento NFA+).

Las diferencias de sensibilidad entre familias también se debe al tipo de daño foliar que desarrollan bajo la exposición al O_3. El daño visible provocado por el contaminante en las leguminosas es en forma de punteaduras necróticas marrón-rojizas en especies de los géneros *Trifolium*, *Biserrula* y *Medicago*; mientras que en las gramíneas fueron pequeñas manchas marrones-rojizas paralelas a los nervios de la hoja en el género *Avena* y en forma de bandeado clorótico en el género *Aegilops*, acompañado en las dos especies por una necrosis apical.

La sensibilidad que muestran las especies en forma de daños visibles permite realizar una primera clasificación de tolerancia al O_3, aunque hay que tener en cuenta que la no presencia de síntomas foliares no es indicativa de resistencia al contaminante, pudiendo estar afectados de forma no visible procesos fisiológicos internos.

El análisis de las proporciones de hojas sanas respecto a senescentes o con daños específicos, así como el grado de exposición necesario para la aparición de los primeros síntomas, permitió establecer una clasificación de sensibilidad para cada familia. El rango de sensibilidad de las especies de la familia *Leguminosae* fue el siguiente: *Trifolium striatum* > *Trifolium cherleri*, *Trifolium glomeratum*, *Trifolium subterraneum*, *Trifolium hirtum* > *Trifolium angustifolium* > *Biserrula pelecinus*, *Medicago minima* > *Anthyllis lotoides*, *Anthyllis cornicina*, *Ornithopus compressus*. La sensibilidad de las especies de la familia *Gramineae* fue: *Avena barbata*, *Avena sterilis*, *Aegilops geniculata* > *Lolium rigidum* > *Briza maxima*, *Aegilops triuncialis*, *Bromus hordeaceus*, *Bromus sterilis* > *Cynosurus echinatus*, *Micropyrum tenellum*, *Vulpia myuros*.

En las especies sensibles, el O_3 afecta distintos procesos a escala bioquímica que afectan al metabolismo celular, repercutiendo a escala fisiológica en la asimilación de carbono (metabolismo primario), y por tanto limitando su capacidad de crecimiento y producción de biomasa (Chaudhary & Agrawal, 2013). Con cierta frecuencia, se ha observado como respuesta generalizada una mayor intensidad de los efectos en el sistema radical respecto al aéreo (Vollsnes et al., 2010), que provoca un aumento de la tasa biomasa aérea/biomasa subterránea, alterando la distribución de carbono entre sus órganos (Andersen, 2003).

Los resultados obtenidos al analizar los distintos parámetros de biomasa en este trabajo demuestran que las leguminosas, en general, fueron más sensibles que las gramíneas también cuando se analizan este tipo de parámetros de crecimiento. La exposición al O_3 provocó efectos negativos en el crecimiento relativo aéreo y radicular en un 70 y 67% respectivamente de las leguminosas estudiadas; sin embargo, sólo un 29% de las gramíneas vieron afectada su biomasa radicular y ninguna su biomasa aérea. El O_3 también alteró la tasa biomasa aérea/biomasa radicular en un 55 % de las leguminosas, mientras que ninguna gramínea se vio afectada.

Cómo en el caso del desarrollo de síntomas foliares, el patrón de respuesta al O_3 observado en las tasas de crecimiento (RGRs) de las especies anuales dependió de la familia (interacción O_3 x familia $p<0.07$): mientras que ambas familias presentaron tasas de crecimiento similares en el tratamiento control sin O_3 (CFA), las RGRs se redujeron intensamente en las leguminosas en relación a las gramíneas para los tratamientos con O_3 NFA y NFA+.

Se estableció un rango de sensibilidad para cada familia basado en la intensidad del efecto del contaminante sobre el crecimiento relativo de la biomasa aérea, radicular y total, tasa biomasa aérea-radicular y parámetros morfológicos (altura de la planta y diámetro), que fue similar al observado considerando el desarrollo de daños foliares. El rango de sensibilidad para las especies de la familia *Leguminosae* fue: *Trifolium striatum, Trifolium subterraneum* > *Trifolium cherleri, Trifolium angustifolium* > *Medicago minima*> *Trifolium glomeratum, Biserrula pelecinus* > *Ornithopus compressus* > *Anthyllis cornicina, Anthyllis lotoides*. Aunque las especies de gramíneas analizadas fueron menos sensibles que las leguminosas, siguiendo el mismo criterio el rango de sensibilidad fue: *Cynosurus echinatus* > *Aegilops geniculata* > *Vulpia myuros,* *Bromus hordeaceus, Bromus sterilis* > *Avena sterilis, Avena triuncialis* > *Briza maxima, Lolium rigidum*.

La heterogeneidad en la respuesta de las especies anuales al O_3 es un indicativo de la capacidad del contaminante para inducir cambios en la composición de los pastos de dehesa, debido a la pérdida de capacidad competitiva de las leguminosas sensibles respecto a las gramíneas relativamente más tolerantes, pudiendo afectar a la estructura y funcionamiento de estos ecosistemas.

Esta disminución de biomasa de leguminosas sensibles podría suponer una reducción de la producción de pasto, pero también de su calidad forrajera, principalmente por la disminución de proteína bruta aportada en gran medida al conjunto del pasto anual por las leguminosas. Además, la disminución del sistema radicular inducida por el O_3 en especies sensibles podría afectar su capacidad de absorción de nutrientes y agua, un efecto que puede ser grave dadas las características de los suelos donde habitan, frecuentemente escasos en nitrógeno y fósforo, y del clima mediterráneo, con frecuente restricciones hídricas en plena estación vegetativa. Estos efectos podrían ser aún mayores en las leguminosas, al considerar el efecto potencial en el desarrollo de los nódulos radicales y la asimilación de nitrógeno atmosférico como han señalado algunos estudios (Hewitt et al. ,2014).

Efecto del ozono en la capacidad reproductiva de tres tréboles mediterráneos anuales considerando el factor competencia

Las plantas anuales terminan su ciclo de vida con éxito cuando consiguen producir la mayor cantidad de semillas viables que pasan a formar parte del banco de semillas del suelo, que es la base de su perpetuación (Peco et al., 2009). Por ello es importante resaltar la importancia del experimento desarrollado en el subcapítulo 3.2. que analizó el efecto del O_3 en la capacidad reproductiva de tres leguminosas del género *Trifolium*.

El comportamiento de los tres tréboles estudiados en este experimento: *Trifolium striatum*, *Trifolium cherleri* y *Trifolium subterraneum* fue muy similar respecto a los dos factores estudiados, exposición al O_3 y competencia. La exposición al O_3 provocó como respuesta general una disminución de la biomasa de flores y semillas. En el análisis combinado de la respuesta de las 3 especies, el contaminante causó una disminución de un 27% y un 34% del peso de flores en los tratamientos NFA y NFA+ respectivamente respecto al control sin O_3. Se trata de un efecto que puede tener consecuencias importantes en la abundancia de las especies sensibles en áreas afectadas de forma crónica por el contaminante.

El análisis de la modulación de la respuesta por la competencia intra e interespecífica fue estudiado considerando en el diseño del experimento mesocosmos donde crecían juntos un trébol y la gramínea *Briza máxima*. En general, la competencia interespecífica (tréboles creciendo en competencia con la gramínea) afectó más al peso de flores en comparación con la competencia intraespecífica (tréboles creciendo en competencia con tréboles). La competición con 1 o 3 plantas de *Briza máxima* provocó respectivamente una reducción del peso de flores de un 23% y 51% cuando se comparó con el valor obtenido en los cultivos de tréboles.

Otro aspecto interesante en cuanto a la capacidad reproductiva de las especies, es el análisis de su capacidad germinativa y de la viabilidad de las semillas. Los resultados para *Trifolium striatum* indican una reducción inducida por el contaminante en el peso de 100 semillas cercana al 10% ($p<0.05$) en plantas crecidas en el tratamiento con mayor O_3 (NFA+). Este efecto en el tamaño de semilla puede ser relevante al tratarse de uno de los caracteres funcionales determinante del tamaño y la supervivencia de las plántulas, especialmente durante su establecimiento, cuando la disponibilidad de agua es fluctuante como sucede con las primeras precipitaciones otoñales, y es un factor clave en la variación interanual en la composición de especies del pastizal (Peco et al., 2009). Además se ha observado que el O_3 provoca un retardo de la germinación debido al aumento de la dureza de la corteza de la semilla que dificulta su germinación. En la actualidad, aunque no ha sido incluido en esta tesis, se están realizando ensayos de germinación y de viabilidad de plántulas procedentes de parentales sometidos a distintos tratamientos de O_3 en colaboración con el Departamento de Ecología de la UAM.

Este tipo de efectos intensifican aún más el efecto del O_3 en la capacidad reproductiva de las especies sensibles que se encuentran en una clara desventaja frente a las más tolerantes. Se pueden derivar importantes implicaciones ecológicas de estos resultados, dado que la composición de estas comunidades depende de la cantidad de semillas de cada especie en el banco de semillas del suelo y su viabilidad (Peco et al., 2009). Los efectos observados en producción de semilla y viabilidad indican la capacidad del contaminante para provocar alteraciones en la estructura y composición de los pastos.

Modulación de la respuesta al ozono de especies pascícolas mediterráneas anuales por la disponibilidad de nitrógeno

Además del incremento de las concentraciones de O_3, existen otros problemas atmosféricos de origen antropogénico con capacidad para modificar los flujos de materia-energía de los ecosistemas y las relaciones de competencia entre los organismos, como el depósito atmosférico de nitrógeno (N). En la actualidad, en extensas zonas rurales de la Península Ibérica, donde la dehesa caracteriza el paisaje, es frecuente la presencia de niveles de O_3 potencialmente tóxicos para la vegetación (Notario et al., 2012, 2013) junto con un depósito de N por encima de los límites para asegurar la estructura de este tipo de vegetación (Rodá et al., 2002; García-Gómez et al., 2014).

Se desarrollaron dos experimentos independientes con el propósito de estudiar el efecto del O_3 y la modulación de la respuesta debida al incremento de la disponibilidad de N (simulado mediante el aporte de nitrato amónico), considerando cinco especies herbáceas anuales, dos gramíneas (*Briza máxima*, *Bromus hordeaceus*) y tres leguminosas (*Trifolium cherleri*, *Trifolium striatum* y *Trifolium subterraneum*), todas ellas características de los pastos de la dehesa. Se consideraron en el análisis los efectos en parámetros de biomasa, capacidad reproductiva, fenología y calidad nutritiva.

Parámetros de biomasa:

Los efectos del O_3 en parámetros de biomasa siguieron un patrón semejante a los observados en los experimentos anteriores realizados con las mismas especies pero solo considerando el factor O_3. En las tres leguminosas se encontró una disminución de su biomasa aérea y, con mayor intensidad, una reducción de su biomasa radical, que provocó una disminución en la tasa biomasa aérea/radicular. El contaminante también produjo una aceleración de la senescencia (incremento de biomasa senescente) que aumentó la tasa biomasa senescente/verde. Las gramíneas tuvieron un comportamiento también similar a los estudios anteriores, puesto que el O_3 incrementó la biomasa senescente y la tasa biomasa senescente/verde en las tres especies. Sin embargo, se observó un efecto positivo singular en relación con el sistema radicular de *Bromus* *hordeaceus*, ya que la biomasa de la raíz aumentó bajo exposiciones elevadas de O_3, disminuyendo su tasa biomasa aérea/radicular. Se trata de una respuesta que podría dar a esta especie una ventaja competitiva frente a las leguminosas sensibles con las que convive, cuyos sistemas radicales pueden verse seriamente afectados por el O_3, poniéndolas en posición de clara desventaja en un hábitat caracterizado por unos recursos nutricionales e hídricos limitados.

La mayor disponibilidad de N incrementó la biomasa de todos los parámetros analizados para las especies de ambas familias. La eficiencia del fertilizante fue menor cuando las condiciones meteorológicas fueron más favorables para el crecimiento de las plantas (las diferencias de biomasa fueron menores entre los distintos tratamientos de N). En general, la respuesta al N de la biomasa aérea fue mayor que la de la biomasa radical, reflejándose en un incremento de la tasa biomasa aérea/biomasa radical por el efecto del fertilizante.

Por tanto, los dos factores analizados, O_3 y N, ejercieron efectos contrarios sobre los parámetros de biomasa: mientras que el O_3 redujo la producción de las especies sensibles, el N aumentó la biomasa. Además, ambos factores tuvieron diferentes dianas prioritarias: el O_3 afectó de forma más intensa a la biomasa radical, mientras que el N estimuló en mayor medida la biomasa aérea. Se encontraron interesantes interacciones que indican que ambos factores se modulan mutuamente, aunque el patrón de respuesta no es generalizable.

En *Trifolium striatum*, los efectos negativos del O_3 en la biomasa aérea total y la biomasa verde se redujo por la mayor disponibilidad de N, aunque solo cuando los niveles de O_3 no fueron muy elevados. También el N compensó parcialmente la reducción de la tasa biomasa verde/senescente en *Trifolium subterraneum*, *Bromus hordeaceus* y *Briza máxima* debido al incremento de la senescencia provocada por el O_3. Sin embargo, en *Trifolium subterraneum* se observó una tendencia contraria, la fertilización nitrogenada intensificó el efecto del O_3 en la biomasa subterránea. En esta misma dirección, el N también incrementó el aumento de biomasa senescente provocada por el O_3 en *Trifolium cherleri*.

Los resultados obtenidos muestran distintas posibilidades en la interacción O_3 x N que depende de la especie, del parámetro afectado y de la intensidad de cada factor. Sin embargo, la respuesta más abundante y robusta ha sido el efecto positivo del N en la reducción de la senescencia inducida por el O_3 observada en tres de las cinco especies estudiadas.

Parámetros de calidad nutritiva:

El O_3 alteró el metabolismo secundario, concretamente la ruta de los fenilpropanoides, como consecuencia parte del carbono asimilado en la fotosíntesis fue derivado hacia la síntesis de lignina, un efecto común con otros estreses bióticos y abióticos. El contenido de lignina (ADL) en la biomasa aérea verde se incrementó en las cuatro especies analizadas: *Trifolium subterraneum*, *Trifolium cherleri*, *Briza máxima* y *Bromus hordeaceus*.

Se observó también un incremento de otros componentes de la pared celular reflejado en los valores de ADF y NDF, excepto en *Bromus hordeaceus*. De acuerdo con estos resultados, la calidad nutritiva expresada en forma del índice RFV disminuyó por efecto del O_3 en los tréboles y en *Briza maxima*, aunque de manera más acusada en los primeros, siendo coherente con su mayor sensibilidad al O_3 de acuerdo a la respuesta observada en biomasa. En el caso de *Bromus hordeaceus*, el análisis de IVDMD y el IVNDFD también disminuyó por la exposición al O_3, lo que podría estar relacionado con el aumento de otros compuestos antinutricionales como compuestos fenólicos. Es interesante considerar, que las gramíneas, aun siendo clasificadas como relativamente tolerantes al O_3 en función de la respuesta de los parámetros de biomasa, sin embargo pasan a ser relativamente sensibles al considerar los parámetros de calidad nutritiva.

El incremento de la concentración de lignina de las paredes celulares podría provocar una cascada de acontecimientos con repercusiones a escala ecosistémica. Los constituyentes de la pared celular son la primera línea de defensa de las plantas frente a patógenos como bacterias, hongos, nematodos e insectos herbívoros; por lo que una mayor lignificación de la pared celular aumentaría la capacidad de protección contra estos estreses bióticos y alterar la cadena trófica. La lignina disminuye la digestibilidad del forraje ya que no puede ser fermentada y además reduce la digestibilidad de otros componentes de la pared celular. Otras repercusiones importantes, podrían ser la alteración de los ciclos de nutrientes y carbono debido a cambios en la tasa de descomposición de los residuos vegetales. Sin embargo, el incremento de lignina tendría un efecto positivo reduciendo las emisiones de metano (CH_4) a la atmósfera. Aunque en esta memoria el único resultado que justifica cambios en el metabolismo secundario es el aumento de lignina, podrían esperarse alteraciones en la concentración de otros componentes fenólicos, que si bien no son requeridos en los procesos primarios de crecimiento y desarrollo, tienen consecuencias en las interacciones de la planta y su ambiente.

El efecto del fertilizante fue contrario al del O_3, ya que el N disminuyó el contenido de fibras (ADF, NDF), pero este efecto solo fue significativo en *Briza máxima*. Como en el caso de los parámetros de biomasa, el efecto de los factores analizados O_3 y N en los parámetros relacionados con la calidad nutritiva del forraje se moduló mediante una interacción entre ambos factores. En este caso la interacción siguió el patrón menos frecuente encontrado en biomasa, ya que el fertilizante intensificó el efecto del O_3 en el contenido de fibras foliares. Esto fue lo que sucedió en *Trifolium subterraneum* donde el aumento de ADF inducido por el O_3 se intensifica al aumentar la disponibilidad de N.

El aporte de N incrementó el contenido de proteína bruta (CP) en los dos tréboles estudiados: *Trifolium cherleri* y *Trifolium subterraneum,* aunque no tuvo efecto en ninguna de las gramíneas estudiadas. Sin embargo, de forma algo sorprendente, en las leguminosas analizadas el O_3 produjo un aumento del CP de las plantas analizadas individualmente. Este resultado podría deberse a un efecto de concentración debido a la reducción de biomasa que también provoca el contaminante en las leguminosas analizadas, aunque también podría relacionarse con una alteración del metabolismo del N en la planta. Sin embargo, hay que considerar que aunque el O_3 provoca un aumento del CP por planta, la CP total del pasto disminuiría por la pérdida de biomasa de leguminosas. Además, el contenido de proteína bruta en *Briza máxima* disminuyó también por el O_3, contribuyendo a la reducción proteica del pastizal. El factor que más determina la calidad de los pastos mediterráneos es la cantidad de materia nitrogenada en el forraje. Por lo tanto, la abundancia de leguminosas es clave por su elevado contenido proteico y por mantener su valor nutricional una vez senescentes. Los efectos negativos del O_3 observados en la calidad nutritiva del forraje, podrían implicar un incremento de la necesidad de aportar suplementos alimenticios para el ganado con el fin de cubrir sus necesidades nutricionales en áreas afectadas de forma crónica por niveles elevados de este contaminante.

Fenología y capacidad reproductiva

El O_3 alteró la fenología de las plantas de *Briza máxima* expuestas a ambientes enriquecidos con O_3, observándose un adelanto de la fase de encañado y una finalización temprana del ciclo vital 12 días antes respecto a las plantas control. Sin embargo, este acortamiento del ciclo no tuvo ninguna repercusión en el peso total de semillas producidas por planta. Las plantas de *Trifolium striatum* expuestas a O_3 también adelantaron la finalización de su ciclo fenológico en 7-11 días según la intensidad de la exposición. Las comunidades diversas como los pastizales anuales mediterráneos, se caracterizan por una compleja complementariedad entre las especies, incluido sus ciclos fenológicos, que permite el aprovechamiento máximo de los recursos nutritivos e hídricos del medio, disminuyendo la competencia entre especies y posibilitando su coexistencia. Sin embargo, los cambios fenológicos inducidos por el O_3 podrían afectar a esta complementariedad, alterando las relaciones de competencia dentro del sistema y afectando a la biodiversidad de la comunidad.

En el caso del *Trifolium striatum,* el acortamiento observado en el ciclo fenológico podría estar relacionado con la reducción del peso de semillas observada en este trébol debido a la exposición al contaminante. Además, para esta especie se detectó una interacción O_3 x N muy interesante relacionada con la capacidad reproductiva. En este trébol, la pérdida de biomasa de flores y semillas debidas a los niveles de O_3, fue contrarrestada por el incremento del fertilizante.

Los resultados relacionados con la capacidad reproductiva de las especies anuales son de una gran trascendencia debido a que este tipo de vegetación utiliza como una única vía de reproducción la semilla, que es crucial para su perpetuación. El O_3 puede afectar al banco de semillas debido a una disminución de la proporción de las semillas de especies sensibles respecto a las tolerantes, provocando a largo plazo cambios en la composición y diversidad del ecosistema.

Los resultados presentados en esta tesis relacionados con la interacción O_3xN, se podrían resumir en dos pautas de respuesta: 1) el N mitigó el efecto del O_3, como se observó en los parámetros de biomasa y capacidad reproductiva de las especies sensibles y 2) el N potenció los daños del O_3, que siguen los parámetros de calidad nutritiva. Además, el sentido de esta interacción dependió de la dosis de N, de la intensidad de la exposición al O_3, del parámetro de respuesta evaluado y de la especie considerada.

Como consideración final de este apartado, es importante señalar que la gran complejidad de estos pastizales hace incierto extrapolar los resultados obtenidos mediante este tipo de diseños experimentales, donde las plantas crecen sin restricción hídrica, en maceta y de forma individual o en mezclas sencillas, a la realidad de las complejas comunidades de pastizal, donde son claves las relaciones de competencia interespecífica e intraespecífica por los escasos recursos de su hábitat, especialmente de agua y nutrientes (Gallardo et al., 2009). Se requiere por ello un importante esfuerzo experimental para abordar estudios de comunidades en condiciones naturales. En el caso de los pastizales mediterráneos de anuales, una siembra pluriespecífica otoñal directamente en suelo en sistemas de fumigación a cielo abierto sería el diseño más adecuado. Sin embargo, la interpretación de los resultados obtenidos en este tipo de experimentos es complicada debido a la gran cantidad de factores no controlados que intervienen espacial y temporalmente, y que pueden llevar a interpretaciones erróneas (Stampfli et al., 2010). Por eso, son importantes los diseños experimentales sencillos como los propuestos en esta memoria, que sin duda contribuirán a comprender e interpretar de forma adecuada futuros experimentos con diseños más complejos.

Propuesta de niveles críticos para ozono en especies pascícolas mediterráneas anuales

Los problemas ambientales y en la salud humana que generan los contaminantes atmosféricos, junto con su amplia distribución regional y continental, más allá de los límites fronterizos de los países, llevó a la necesidad de establecer un programa a escala internacional para desarrollar políticas y estrategias de gestión medioambiental para el control de la contaminación atmosférica. Para ello, en 1979 se desarrolló desde la Comisión Económica para Europa de Naciones Unidas el Convenio sobre Contaminación Atmosférica Transfronteriza a Gran Distancia (*Convention on Long-Range Transboundary Air Pollution*, CLRTAP/UNECE), firmado actualmente por 51 países, entre ellos España y la UE con entidad propia. Este programa adoptó la metodología de niveles/cargas críticas (valores umbrales) para establecer la sensibilidad de los receptores a los contaminantes y desarrollar estrategias de reducción de la contaminación que eviten la superación de estos valores límite.

Los niveles críticos (NCs) para el O_3 (CL_e, *critical level* en sus siglas en inglés) se definen como la exposición acumulada de O_3 (CL_{ec}) o el flujo estomático de O_3 (CL_{ef}) por encima del cual se producen efectos adversos sobre la vegetación sensible de acuerdo al conocimiento actual (CLRTAP, 2010). Los primeros NCs se establecieron en 1988, y son revisados periódicamente para incorporar los conocimientos adquiridos para los distintos tipos de vegetación europea en base a los proyectos de investigación que se desarrollan de forma continua. Los NCs definidos en el Convenio se emplean para realizar análisis de riesgo de efectos provocados por O_3 a escala europea y evaluar las políticas ambientales de calidad de aire, y además son la base de los valores objetivo establecidos en la directiva Europea de Calidad del Aire para la protección de la vegetación (2008/50/CE).

El conjunto de los cinco experimentos realizados en la presente tesis han generado una extensa base de datos sobre la respuesta de las herbáceas anuales al O_3. El análisis conjunto de esta base de datos ha permitido la construcción de las funciones de exposición y dosis-respuesta al O_3, a partir de las que se han propuesto NCs para la protección de este tipo de vegetación siguiendo la metodología del CLRTAP/UNECE (CLRTAP, 2010). Las funciones que cuantifican los efectos del O_3 indican que la intensidad de la respuesta al O_3 está más relacionada con los flujos de absorción estomática al interior de la planta a través de los estomas que con la concentración del contaminante en la atmósfera.

Los parámetros considerados para la definición de NCs, se han seleccionado en base a la incidencia en los principales servicios ecosistémicos de los pastos y en criterios estadísticos relacionados con la robustez de las funciones calculadas. Por ello, se han seleccionado tres variables para establecer los NCs correspondientes: biomasa total aérea (producción), CFV (calidad forrajera), y producción de flores y semillas (biodiversidad). Además, se ha considerado un efecto del 10% respecto al tratamiento CFA para definir los NCs, en concordancia con los establecidos para otras comunidades de vegetación semi-natural perenne (CLRTAP, 2010). La robustez del análisis estadístico permite proponer como definitivos CLe_c y CLe_f para biomasa y capacidad reproductiva; mientras que se consideran valores provisionales los correspondientes a calidad nutritiva, ya que se requiere un mayor número de experimentos para alcanzar su validez como NCs definitivos (tablas 4.1, 4.2, 4.3).

Los NCs están por definición orientados a la protección de las especies más sensibles (CLRTAP, 2010), por lo que la elaboración de las relaciones exposición y dosis respuesta se han desarrollado considerando sólo las especies sensibles para cada parámetro. Por ello, los niveles críticos propuestos deben ser destinados para la valoración de riesgos, ya que su empleo para cuantificación de daños llevaría a una sobreestimación de los efectos derivados del O_3.

Parámetro	**Ensayos**	**n**	**Familia (num. spp.)**	**Ecuación**	**Intervalo de confianza**	**R^2**	**p**
Biomasa aérea	5	51	Legum. (7)	-0.0032x+100	(-0.0038, -0.0026)	0.41	<0.001
CFV	1	9	Legum. (2) Grami. (1)	-0.0044x+100	(-0.0063, -0.0025)	0.57	<0.001
Peso de flores y semillas	3	15	Legum. (3)	-0.0050x+100	(-0.0065, -0.0035)	0.45	<0.001

*la ordenada en el origen de la recta ajustada al 100

Tabla 4.1. Funciones exposición-respuesta considerando el índice de concentración de O_3 acumulado AOT40. Legum=Leguminosas; Grami=Gramíneas.

Parámetro	Ensayos	n	Familia (num. spp.)	Ecuación	Intervalo de confianza	R^2	p
Biomasa aérea	5	51	Legum. (7)	-1.0276x+102.5	(97.2, 107.8) (-1.26, -0.079)	0.60	<0.001
CFV	1	9	Legum. (2) Grami. (1)	-3.004x+103.8	(95.8, 111.9) (-3.82, -2.19)	0.90	<0.001
Peso de flores y semillas	3	15	Legum. (3)	-2.0506x+104.8	(87.5, 104.8) (-2.88,-2.05)	0.69	<0.001

Tabla 4.2. Funciones dosis-respuesta considerando el índice de absorción de O_3 acumulado POD1. Legum=Leguminosas; Grami=Gramíneas.

Índice	Biomasa aérea	CFV	Peso de flores y semillas
AOT40 (ppb h)	3116 (2631, 3846)	2272 (1587, 4000)	1998 (1538, 2857)
POD1 (mmol m^{-2})	12.18 (8.88, 15.47)	4.60 (2.71, 6.50)	7.22 (1.10, 13.34)

Tabla 4.3. Niveles críticos de O_3 e intervalos de confianza entre paréntesis para pastizales de especies anuales, para un período de acumulación de 45 días. Legum=Leguminosas; Grami=Gramíneas.

5. CONCLUSIONES GENERALES

El O_3 causó la aparición de daños visibles específicos y daños no específicos relacionados con una aceleración de la senescencia foliar. Ambos efectos fueron observados en leguminosas y gramíneas.

El O_3 afectó al metabolismo primario de las leguminosas sensibles, provocando: reducción de biomasa verde aérea, total aérea (suma de verde y senescente), radicular y total (suma de aérea y radical). El efecto del O_3 fue mayor en la biomasa radicular que en la biomasa aérea, causando un incremento de la tasa biomasa aérea/radical. La biomasa de las gramíneas en general no fue afectada por el O_3.

La exposición al O_3 modificó la fenología en ambas familias, acelerando la floración y acortando el ciclo de vida.

El O_3 afectó a la capacidad reproductiva de especies del género *Trifolium* disminuyendo la producción de flores, la producción de semillas y el peso de la semilla.

Los efectos en la fenología y capacidad reproductiva están considerados dentro de los denominados “efectos memoria” que se producen con posterioridad a la exposición al contaminante debido a los daños sufridos en su metabolismo.

El O_3 alteró el metabolismo secundario incrementando el contenido de lignina de la biomasa aérea en ambas familias, siendo sustancialmente mayor en las leguminosas. El O_3 también aumentó las concentraciones de ADF y NDF, con consecuencias en una disminución de la calidad nutritiva reflejada en el índice RFV.

El O_3 incrementó la proteína bruta en las plantas de leguminosas, sin embargo en gramíneas se observó una disminución o ningún efecto.

Las leguminosas anuales pueden considerarse más sensibles al O_3 cualitativamente y cuantitativamente respecto a las gramíneas anuales. El género *Trifolium* fue el más sensible, mientras que *Ornithopus y Anthyllis* fueron más tolerantes, lo que refleja un rango amplio de sensibilidad al contaminante de las especies de una misma familia.

La mayor disponibilidad de N en el sustrato favoreció de forma general la producción de biomasa en todas las especies. El efecto fue más intenso en el sistema aéreo respecto al radicular (alterando la tasa biomasa aérea/radicular) y más eficiente cuando las condiciones de crecimiento fueron más desfavorables. El aporte de N incrementó la CP en las leguminosas, sin afectar a las gramíneas.

Los efectos del O_3 se modularon por la disponibilidad de N, sin embargo no se encontró un patrón generalizable en la interacción, ya que dependió de la especie, el parámetro considerado, los niveles de ambos factores y las condiciones ambientales.

La intensidad de la respuesta al O_3 estuvo más relacionado con los flujos de absorción estomática que con la concentración del contaminante en la atmósfera.

Se proponen CL_{ec} y CL_{ef} de O_3 para comunidades pascícolas mediterráneas con predominio de especies anuales para las variables de respuesta biomasa total aérea y producción de flores y semillas.

Los resultados de la presente memoria indican que el O_3 debe ser considerado como un factor de estrés para el desarrollo de los pastizales anuales en el marco del cambio global.

GLOSARIO

Abreviaturas empleadas en el texto:

AA: *ambient air* (aire ambiente).

ADF: *acid detergent fiber* (fibra ácido detergente).

ADL: *acid detergent lignin* (lignina ácido detergente).

AOT: *Accumulated exposure Over a given Threshold* (concentración acumulada de O_3 por encima de un determinado umbral).

AOT40: *Accumulated exposure Over a Threshold ozone concentration of 40 ppb* (concentración acumulada de O_3 por encima de 40 ppb).

CIEMAT: Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas.

CFA: charcoal filtered air (aire filtrado con carbón activo).

CFCs: clorofluorocarbonados.

CFV: *consumable food value* (valor alimenticio para consumo).

CP: *crude protein* (proteína bruta).

CL_e: *critical level* (nivel crítico).

CL_{ec}: *critical level* (nivel crítico basados en exposición acumulada de O_3).

CL_{ef}: *critical level* (nivel crítico basados en flujo estomático absorbido acumulado de O_3).

CLRTAP/UNECE: *Convention on Long-Range Transboundary Air Pollution* (Convenio de Naciones Unidas sobre Transporte Transfronterizo a Gran Distancia de Contaminantes Atmosféricos).

COVs: compuestos orgánicos volátiles.

COVNMs: compuestos orgánicos volátiles no metánicos.

DO_3SE: *Deposition of Ozone and Stomatal Exchange* (depósito de ozono e intercambio estomático).

EDU: etilén-diurea.

EEA: European Environment Agency (Agencia Europea de Medio Ambiente).

EMEP: *European Monitoring and Evaluation Programme* (Programa sobre seguimiento en continuo y evaluación del transporte atmosférico de contaminantes).

ENA: *European Nitrogen Assessment* (Evaluación del nitrógeno en Europa).

EROs: especies reactivas de oxígeno.

f_{light}: *modifying function depending on light* (modificación del módelo en función de la luz).

f_{min}: relative mínimum stomatal conductance (conductancia estomática mínima relativa).

f_{O3}: *modifying function depending on ozone* (modificación del módelo en función de la concentración de ozono).

f_{phen}: *modifying function depending on phenology* (modificación del módelo en función de la fenología).

f_{SWP}: *modifying function depending on soil water pressure* (modificación del módelo en función de la presión de agua en el suelo).

f_{temp}: *modifying function depending on temperature* (modificación del módelo en función de la temperatura).

f_{VPD}: *modifying function depending on vapour pressure déficit* (modificación del módelo en función del déficit de presión de vapor).

g_s: conductancia estomática.

g_{max}: conductancia estomática máxima.

HCNMs: hidrocarburos no metánicos.

ICPs: *International Cooperative Programs* (Programas de cooperación internacional).

ICP- *Forests*: *International Cooperative Programme on Assessment and Monitoring of Air Pollution Effects on Forests* (Programa de Cooperación internacional para la evaluación y seguimiento de los efectos de la contaminación atmosférica en los bosques).

ICP-*Integrated Monitoring*: *International Cooperative Programme on Integrated Monitoring of Air Pollution Effects on Ecosystems* (Programa de Cooperación Internacional para la integración de la evaluación y seguimiento de los efectos de la contaminación atmosférica en los ecosistemas).

ICP-*Vegetation*: *Internacional Cooperative Programme on Effects of Air Pollution on Natural Vegetation and Crops* (Programa de Cooperación Internacional de los efectos de la contaminación atmosférica en vegetación seminatural y cultivos).

IVDMD: *in vitro dry matter digestibility* (digestibilidad in vitro de materia seca).

IVNDFD: *in vitro NDF digestibility* (digestibilidad in vitro de NDF).

MAGRAMA: Ministerio de Agricultura, Alimentación y Medio Ambiente.

NCLAN: *National Crop Loss Assesment Network* (Programa nacional para la valoración de pérdidas en cultivos, EEUU).

NC: nivel crítico.

NDF: *neutral detergent fiber* (fibra neutro detergente).

NFA: *non-filtered air* (aire no filtrado).

NFA+: *non filtered air plus* (aire no filtrado suplementado).

OTCs: *Open Top Chambers* (cámaras de techo descubierto).

PLA: *Projected leaf área* (área de hoja proyectada).

PODy: *Phytotoxic Ozone Dose* (dosis de ozono fitotóxica).

PAN: peroxiatil nitrato.

PAR: *Photosynthetic active radiation* (radiación fotosintéticamente activa).

R: suma de todas las resistencias.

R_a: resistencia aerodinámica.

R_b: resistencia de la capa límite.

R_c: resistencia de superficie.

R_{ext}: resistencia reticular.

R_s: resistencia estomática.

RFV: *Relative feed value* (valor relativo del forraje).

RGR: *relative growth rate* (tasa de crecimiento relativo).

SCI: *Science Citation Index* (índice de citas científicas).

UNECE: *United Nations Economic Commission for Europe* (Consejo Económico para Europa de Naciones Unidas).

US EPA: *United States, Environmental Protection Agency* (Agencia de protección ambiental de Estados Unidos).

VPD: *vapour pressure déficit* (déficit de presión de vapor).

WGE: *Working Group on Effects* (Grupo de Trabajo sobre Efectos).