FLUJOS ESTOMÁTICOS DE OZONO Y SUS EFECTOS SOBRE LA VEGETACIÓN. RELACIONES DOSIS RESPUESTA

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Madrid, 2009

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1. INTRODUCCIÓN Y OBJETIVOS

1. INTRODUCCIÓN GENERAL

Desde la revolución industrial, las emisiones de gases que se derivan de las actividades humanas han provocado y siguen provocando cambios capaces de alterar los complejos equilibrios de la atmósfera que mantienen las propiedades y funciones de la atmósfera. Estos cambios pueden deberse a la modificación de la proporción de sus componentes o mediante la introducción de elementos extraños a ésta, lo que conocemos en su conjunto como contaminación atmosférica. La contaminación atmosférica supone un problema ambiental que puede tener efectos sobre multitud de receptores (salud o bienestar humano, bienes y medioambiente en su conjunto) y que además actúa a distintas escalas, desde cambios locales de la calidad del aire hasta procesos globales.

Los efectos de la contaminación atmosférica sobre el medio ambiente suelen manifestarse en primer lugar en las especies que se encuentran en los primeros eslabones de la red trófica: los productores primarios. Esto tiene implicaciones muy importantes para el funcionamiento del ecosistema, al producirse una alteración de los flujos de materia y energía propios del mismo. No es de extrañar que la mayor parte de bibliografía científica relacionada con los efectos de la contaminación atmosférica sobre los ecosistemas se centre en las perturbaciones que ésta produce en la biología de los vegetales. En el caso concreto del ozono, los primeros estudios sobre los efectos fitotóxicos del ozono datan de finales de los años 1950. Desde entonces se han realizado numerosas investigaciones para determinar el impacto del ozono sobre los receptores vegetales.

El ozono (O_3) es un compuesto presente de forma natural en la atmósfera tanto en la estratosfera, donde se encuentra el 80% del O_3 atmosférico, como en la troposfera. El O_3 troposférico, al igual que otros oxidantes, no se emite directamente desde ninguna

fuente, sino que se forma en el aire a partir de series complejas de reacciones de carácter fotoquímico a partir de sus precursores. Las principales fuentes antropogénicas de precursores del O_3 son la actividad industrial y el transporte, aunque también existen fuentes naturales. Cuando las condiciones son favorables, el O_3 se acumula en la troposfera hasta alcanzar concentraciones que pueden provocar efectos tóxicos sobre la salud humana, los materiales, el crecimiento y fisiología de las plantas así como el funcionamiento de los ecosistemas. Estos efectos se traducen hoy en día en problemas a escala local, regional e internacional (Ashmore, 2005). Los efectos de este contaminante en las plantas, los receptores más sensibles, pueden manifestarse como daños visibles en las hojas, reducción del crecimiento y la producción de las plantas, caída acelerada del follaje y alteraciones en la sensibilidad frente a otros estreses como la sequía, las plagas, etc. Se han documentado daños sobre numerosos cultivos agrícolas y hortícolas y también sobre especies de árboles y de herbáceas que conforman los pastizales.

La necesidad de establecer políticas y estrategias de gestión medioambiental que controlen la contaminación atmosférica han motivado, durante las últimas tres décadas, el estudio de los procesos implicados en la formación del O_3 y sus efectos sobre los receptores vegetales y la salud humana. El objetivo último de estos estudios consiste en determinar los límites de la concentración de O_3 que no deben ser superados para garantizar la integridad de los seres vivos (niveles críticos) y establecer estrategias de reducción de la contaminación que evitarían la superación de los niveles críticos. Con base en este tipo de estudios se han establecido diversos protocolos basados en la metodología de cargas y niveles críticos. En este sentido, el Convenio de Ginebra sobre Transporte Transfronterizo a Larga distancia de la Contaminación Atmosférica (CLRTAP, 1979) de la Comisión Económica para Europa de la ONU (UNECE), a través de los protocolos derivados del mismo, como el Protocolo de Gotemburgo (Gothenburg, 1999), ha establecido niveles críticos de O_3 para la protección de la vegetación y la salud humana basados en la concentración, según se recoge en la legislación europea (Directiva 2008/50/CE). Los protocolos que establecen los niveles críticos se revisan y modifican periódicamente tomando en consideración los resultados de los trabajos científicos como los que se presentan en esta tesis. Por ejmplo, en los últimos años se ha documentado cómo las condiciones ambientales pueden modular la toxicidad del O_3 para las plantas. Una misma exposición (concentración por tiempo) puede provocar efectos divergentes en función de características ambientales tales como

la humedad del aire y del suelo, la disponibilidad de nutrientes en el suelo o la presencia de especies competidoras. Por este motivo se hace necesario el establecimiento de nuevos niveles críticos basados en la dosis de ozono absorbido por la planta, tal como se recoge en la última revisión del CLRTAP (CIAM, 2007). En este último punto es en el que se integran los trabajos presentados en esta memoria, que han contribuido en los debates para el establecimiento de niveles críticos basados en el flujo absorbido de O_3 en el marco del *Internacional Cooperative Programme on Effects of Air Pollution on Natural Vegetation and Crops* (ICP-Vegetation) del CLRTAP.

2. EL OZONO TROPOSFÉRICO

El ozono (O_3) es una forma alotrópica del oxígeno, presente de forma natural en la atmósfera. Su concentración presenta una marcada distribución vertical: mientras que el 90% del O_3 se sitúa en la estratosfera, alrededor de los 25 km. de altura, lo que se conoce comúnmente como 'la capa de ozono', un 10% se localiza entre la superficie terrestre y los 10 km. de altitud (Seinfield y Pandis, 2006), región que se conoce como troposfera. Es por ello que éste último se conoce como O_3 troposférico.

Los niveles de O_3 registrados en la troposfera son el resultado de un complejo equilibrio entre los procesos de formación, transporte y destrucción, todos ellos influidos a su vez por una combinación de factores meteorológicos y fotoquímicos. Entre ellos, el transporte desde la estratosfera y la formación fotoquímica mediante reacciones a partir de sus precursores, son las fuentes más importantes que elevan la concentración de O_3 en la troposfera (Royal Society, 2008). Los precursores más importantes son los óxidos de nitrógeno (NO_x), los compuestos orgánicos volátiles (COV), el metano (CH_4) y el monóxido de carbono (CO). La combinación de los procesos mencionados contribuyen a crear un nivel de fondo, estimado en 20-45 ppb[1] para latitudes medias del hemisferio norte (Vingarzan, 2004), de los cuales entre 7 y 14 ppb son atribuibles a la inyección desde la estratosfera (Monks, 2000), aunque estos valores varían estacional y latitudinalmente.

En una situación de atmósfera contaminada en la que las emisiones de precursores, principalmente NO_X y COV, y las reacciones fotoquímicas elevan la

[1] ppb, parte por billón = nmol mol^{-1}

concentración de O_3 por encima del fondo, se pueden alcanzar valores de hasta 200 y 400 ppb (Emberson *et al.*, 2003; Fiala *et al.*, 2003; WHO, 2006; Royal Society, 2008), generalmente más elevados en áreas suburbanas y rurales y zonas montañosas que en el centro de las ciudades (NEGTAP, 2001). En zonas urbanas con emisiones intensas de NO_x, el O_3 se mantiene en niveles relativamente bajos en comparación con los alrededores, aunque la inyección de COV puede producir picos de concentración. En áreas rurales, alejadas de las fuentes de emisión de contaminantes atmosféricos, el transporte del propio O_3 o de sus precursores, así como las emisiones naturales de COV por parte de la vegetación y el suelo hacen del O_3 un importante contaminante en este medio (Ashmore, 2005). Las zonas montañosas también experimentan, generalmente, niveles elevados debido al aumento de la concentración de O_3 con la altitud. Esta situación se ha descrito en numerosas áreas de montaña (Alonso y Bytnerowicz, 2003; Ribas y Peñuelas, 2006; Alonso *et al.*, 2009). Los aumentos se relacionan con incrementos en la intensidad de radiación solar, que favorece las reacciones fotoquímicas, con la disminución de los procesos de destrucción del O_3, con aumentos de los niveles de fondo debido a procesos fotoquímicos que ocurren a escala regional y sinóptica y con el intercambio de gases con las capas más altas de la troposfera (véase revisión en Alonso y Bytnerowicz, 2003).

El O_3 no sólo supone un problema a escala local, sino que constituye un problema de contaminación atmosférica a escala regional y global. Esto es debido a dos circunstancias: el O_3 puede formarse a partir de sus precursores largo tiempo después de que éstos hayan sido emitidos; además, tanto el O_3 como sus precursores pueden ser transportados a grandes distancias. Entre 20 y 40 ppb de O_3 entran en Europa desde el oeste originados por precursores emitidos fuera de este territorio (Collins *et al.*, 2000). A su vez, las emisiones procedentes de Europa y Asia contribuyen a elevar las concentraciones de O_3 que se registran en Norteamérica (Royal Society, 2008).

En las secciones siguientes se revisan brevemente los procesos de formación y destrucción del ozono troposférico y las tendencias de su concentración en relación con otros componentes del cambio global. Estos procesos determinan el régimen presente y futuro de exposición al O_3 de los cultivos y los ecosistemas, lo que tiene muchas implicaciones en cuanto a sus efectos fitotóxicos y la efectividad de los mecanismos de control de la contaminación que se explican en los apartados posteriores.

2.1. Formación del ozono troposférico.

La concentración de O_3 varía localmente en función del equilibrio entre los procesos de formación y destrucción. Los principales procesos químicos implicados necesitan de la presencia de radicales libres, principalmente formados en la fotólisis del propio O_3. Además, es necesaria la presencia de otros compuestos como COV, CH_4, CO, NO_x y, por tratarse de reacciones fotoquímicas, de radiación solar. También deben darse unas condiciones de estabilidad atmosférica (ausencia de viento y lluvias) y temperaturas moderadas. Estas características provocan que la concentración de O_3 dentro de una misma localidad varíe de forma diaria, estacional e interanual, aunque la comprensión de algunos aspectos de la química del O_3 todavía está en desarrollo. Así mismo, no existe una relación lineal entre la concentración de precursores y la formación de O_3.

El proceso se inicia con la fotólisis del O_3 al interaccionar con la radiación solar de longitud de onda inferior a los 320 nm (eq. 1). En presencia de agua, los átomos de oxígeno (O) reaccionan generando radicales hidroxilo (OH), o pueden volver a formar O_3 después de interaccionar con una molécula inerte, normalmente nitrógeno (N_2). La eficiencia de generación de radicales hidroxilo es mayor cuando más alta es la temperatura.

Los radicales OH juegan un papel central en la química del O_3 troposférico. Estos OH reaccionan con el CH_4, el CO o los COV para iniciar ciclos de reacciones que generan y eliminan el O_3. El resultado neto de estos ciclos depende de la concentración de NO_x y de la presencia de compuestos orgánicos en el aire. En general se distinguen 3 situaciones en función de la concentración de NO_x: baja (del orden de ppt [2]), intermedia o alta. El ciclo completo se muestra en la figura 1.1 para cada una de las 3 situaciones:

- Cuando la concentración de NO_x es baja, inferior a las 20 ppt como las que se registran en la región del Pacífico sur, aunque es una situación que también ocurre en grandes zonas de la troposfera, se produce una eliminación neta de O_3 (figura 1.1a). Los radicales OH formados según la ecuación 1, reaccionan con el CO y el CH_4 para formar metil-hidroperóxido (CH_3OOH) y peróxido de hidrógeno (H_2O_2) que actúan como sumideros y no regeneran el O_3 consumido en la producción de OH. También actúa aquí

[2] ppt, parte por trillón.

una segunda vía de eliminación del O_3 en la que intervienen radicales OH_2 para regenerar el OH consumido en la oxidación del CO.

- Bajo concentraciones intermedias de NO_x, como las que se registran en las zonas rurales de los países industrializados, el ciclo conduce a una producción neta de O_3 (figura 1.1b). La concentración de O_3 aumenta con la de NO_x, por lo que se denomina a este ciclo, 'limitado por NO_x'. Al aumentar la concentración de estos compuestos, las reacciones de conversión del NO a NO_2 compiten mejor con la formación de CH_3OOH y H_2O_2 y con el consumo de O_3 por parte de la conversión HO_2 a OH. Como resultado, el NO_2 acumulado se disocia por fotólisis con radiaciones de longitud de onda menor a 420 nm, y genera nuevo O_3 mediante el proceso descrito en la ecuación 2.

- Cuando la concentración de NO_x aumenta hasta niveles propios de zonas cercanas a puntos de emisión o del centro de las grandes ciudades, se produce una inhibición de la formación de O_3. Las reacciones de conversión de NO a NO_2 dominan en el ciclo y este NO_2 reacciona con los OH para formar ácido nítrico (HNO_3) (figura 1.1c). El HNO_3 se convierte en el principal sumidero de los radicales OH, que no entran en el ciclo de formación de O_3. En este caso la formación de O_3 se denomina 'limitada por COV', pues la presencia de estas sustancias reactiva la formación de O_3. En los ambientes urbanos, las concentraciones de NO, NO_2 y O_3 se encuentran acopladas en un ciclo nulo (ecuación 3). Este ciclo puede disminuir mucho las concentraciones de O_3 cuando aumentan las emisiones de NO. Sin embargo, en estas circunstancias se pueden producir fuertes episodios de O_3 cuando los COV son inyectados en una atmósfera enriquecida en NO_x.

Es importante considerar en el ciclo descrito anteriormente que existen otros procesos de generación secundaria de radicales libres a través de la oxidación del formaldehído (HCOH), que aparece como sumidero en las reacciones anteriores (figura 1.1). Esta oxidación regenera radicales HO_2 y produce CO, cuyo papel en el ciclo de formación o destrucción del O_3 ya se ha descrito.

$$O_3 + hn \rightarrow O + O_2$$
$$O + H_2O \rightarrow 2OH \qquad (1)$$
$$O + O_2 + M \rightarrow O_3 + M$$

$$NO_2 + hn \rightarrow NO + O$$
$$O + O_2 + M \rightarrow O_3 + M \qquad (2)$$

$$NO_2 + hn \rightarrow NO + O$$
$$O + O_2 + M \rightarrow O_3 + M \qquad (3)$$
$$NO + O_3 \rightarrow NO_2 + O_2$$

Una vez formado, el O_3 tiene un tiempo de residencia medio en la atmósfera estimado en 22 días (Stevenson *et al.*, 2006), aunque en zonas cercanas a la superficie, los procesos de eliminación reducen el tiempo de residencia a tan solo 1 ó 2 días.

2.2. Eliminación del ozono de la atmósfera: depósito seco sobre superficies terrestres

En las ciencias atmosféricas, el flujo de un gas traza o de material particulado que produce su eliminación de la atmósfera hacia una superficie se denomina depósito (Seinfield y Pandis, 2006). Cuando este depósito se realiza en ausencia de precipitaciones, se denomina depósito seco.

Entre los procesos que eliminan el O_3 de la atmósfera destacan, por su importancia a escala global (especialmente en el hemisferio sur y en latitudes altas), las reacciones catalizadas por compuestos halogenados que ocurren sobre los océanos. Los halógenos más importantes implicados son el Bromo (Br) y el Yodo (I), que se liberan por fotodisociación de los compuestos orgánicos e inorgánicos emitidos por el mar o presentes en los aerosoles marinos. El otro mecanismo de eliminación del O_3 troposférico es el depósito seco sobre la superficie.

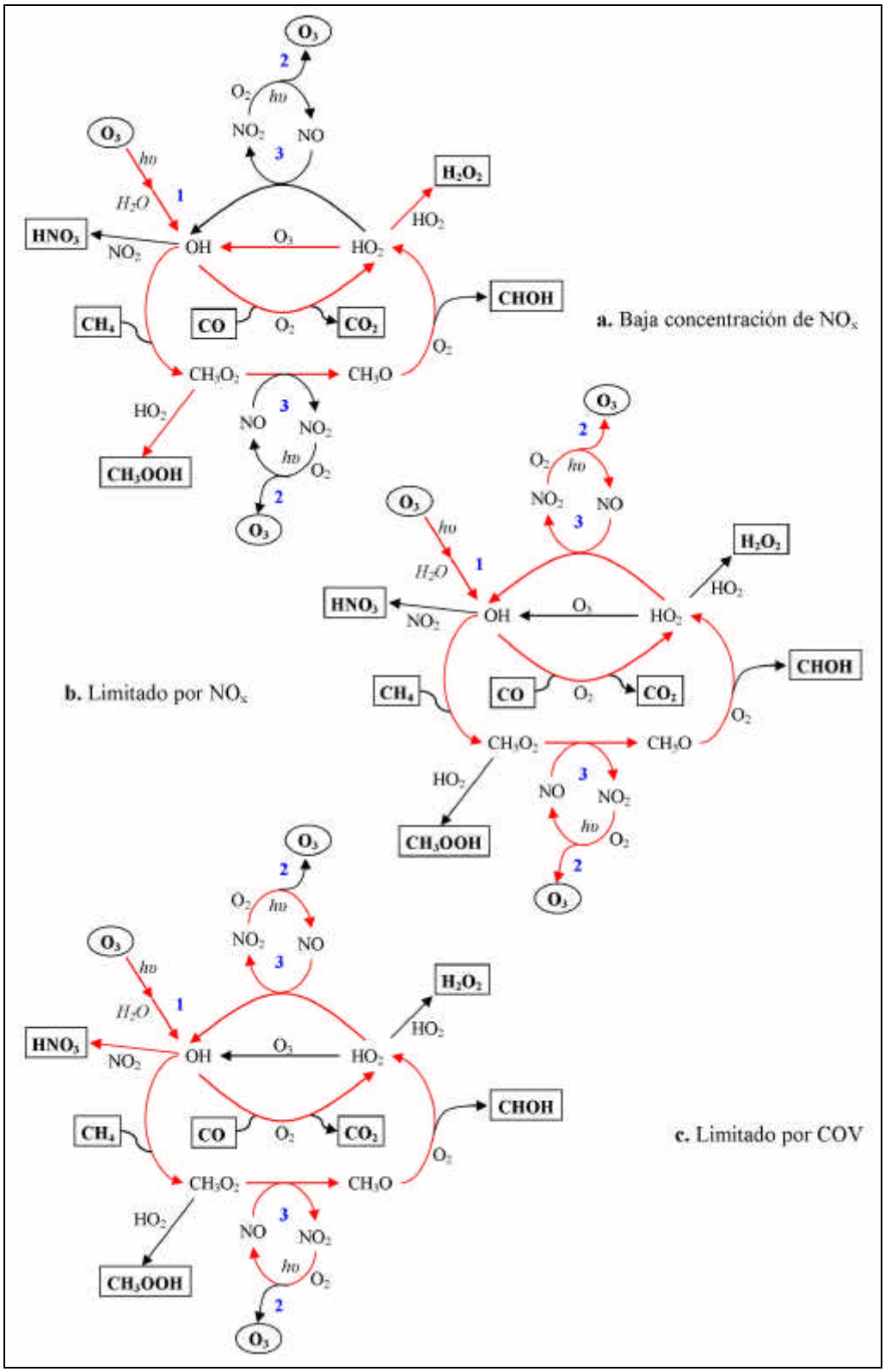

Figura 1.1. Representación de los procesos de formación y destrucción de O_3 mediante la oxidación atmosférica de CO y CH_4 a partir de radicales libres en tres situaciones: baja concentración de óxidos de nitrógeno (NO, NO_2) (a), concentración intermedia de óxidos de nitrógeno (b) y concentración elevada de óxidos de nitrógeno (c). Los COV producen reacciones análogas a las del CH_4. Las líneas en rojo representan los procesos dominantes en cada situación. Las moléculas encuadradas representan los sumideros donde terminan los radicales libre sus ciclos de reacciones. *h*?, luz solar. Los números se corresponden con las reacciones representadas en las ecuaciones 1-3 (Adaptada de Royal Society, 2008).

El O_3 es un gas muy reactivo que se deposita rápidamente sobre todas las superficies: suelo, vegetación, materiales de construcción y la superficie del agua. El depósito seco resulta de la reacción de O_3 con las superficies del suelo y la vegetación y de su absorción por parte de los estomas de las plantas. Las tasas de depósito seco de una sustancia dependen de variables meteorológicas, de las propiedades de la superficie y de las características propias de una sustancia así como de la interrelación entre estos factores. En el caso concreto de la vegetación algunas propiedades están ligadas a la actividad fisiológica de la planta, por ejemplo la apertura estomática. Este parámetro está influido a su vez por una serie de factores que la gobiernan como la velocidad del viento, la turbulencia y la presencia de agua sobre las superficies. Además, la composición del aire que rodea las plantas está influida por los gases que la propia vegetación emite, como COV de origen biogénico, además de características micro-meteorológicas diferentes, lo que puede traducirse en cambios en la concentración de O_3 debido a reacciones químicas.

La vía principal de depósito de O_3 sobre la vegetación causante de daños fisiológicos son los estomas. Como se explica más adelante, el proceso de depósito seco y absorción estomática del ozono se cuantifica en los estudios de efectos del O_3 sobre la vegetación utilizando el modelo de resistencias (Gaastra, 1959).

2.3. Ciclos temporales de la concentración de ozono

Todas las reacciones vistas anteriormente son moduladas por factores meteorológicos y las emisiones de los precursores de la zona. Esto condiciona la existencia de una variación espacial y temporal en los niveles de O_3 troposférico.

Las reacciones de formación del ozono, al tratarse de procesos fotoquímicos, son altamente dependientes de la presencia e intensidad de la radiación solar. También se ven afectados, como hemos visto, por otros factores como la temperatura o la estabilidad atmosférica. Estas características determinan una marcada variación diurna, en la que los mayores valores diarios se registran hacia mediodía, coincidiendo con las mayores intensidades de radiación solar, mientras que los niveles mínimos se registran durante la noche.

Sin embargo, los perfiles diarios cambian según la zona considerada en relación con el volumen de precursores emitidos, el transporte de la contaminación hacia o desde otros lugares y la topografía. Los perfiles de O_3 registrados en zonas montañosas, por ejemplo, presentan valores más elevados y una menor oscilación diaria que los registrados en el fondo de los valles, debido a las diferencias en las tasas de formación y depósito entre ambas zonas y a la influencia del viento en el transporte de precursores.

En el caso de las zonas rurales, los procesos de transporte atmosférico desde otras zonas emisoras de precursores tienen gran influencia sobre el perfil de O_3. Durante la noche se pueden producir inversiones térmicas que separan las capas del aire. La que está por debajo de la inversión, en contacto con el suelo, reduce lentamente los niveles de ozono por reacciones con otras sustancias y por el depósito seco sobre la superficie. En cambio, la capa superior se encuentra enriquecida en contaminantes debido a los procesos de transporte. Durante la mañana, al romperse la inversión como consecuencia del calentamiento del suelo, la capa enriquecida se mezcla con la capa inferior, lo que provoca que la concentración de O_3 se incremente muy rápidamente durante las primeras horas del día. En aquellos lugares en los cuales la emisión de percusores sea mínima y por lo tanto los procesos fotoquímicos poco intensos, los niveles de ozono registrados presentan perfiles diarios casi constantes alrededor de un valor. Este patrón también se registra en aquellos lugares afectados por procesos de transporte de contaminantes.

Además de los ciclos diarios, la concentración de ozono también cambia según la época del año. Así se ha detectado que en estaciones remotas, no afectadas directamente por fuentes de contaminantes, existe un ciclo anual cuyo máximo se sitúa a finales del invierno o principios de la primavera. Estos niveles se mantendrán altos, debido al aumento de las temperaturas y de la radiación solar, durante el verano hasta que vuelvan a decrecer en invierno. El máximo primaveral podría explicarse como consecuencia de la acumulación de percusores que no reaccionan en invierno, debido a las bajas temperaturas y a la escasez de radiación solar. También interviene en este máximo la intrusión de ozono desde la estratosfera. En zonas afectadas por fuentes de contaminación del aire, las máximas concentraciones ocurren en primavera y verano.

2.4. Distribución geográfica del ozono

Las reacciones de formación y eliminación del O_3 en la atmósfera, condicionadas por multitud de factores climáticos y topográficos, la intensidad de la radiación solar y la concentración de precursores determinan su distribución geográfica. En general aquellas zonas rurales, costeras y montañosas próximas a fuentes emisoras de precursores son los lugares con mayores concentraciones de O_3. En las ciudades y en puntos próximos a las fuentes de emisión, las concentraciones elevadas de NO_x determinan una situación 'limitada por COV' que mantiene niveles moderados de O_3. En cambio, en zonas rurales próximas a estas fuentes de contaminación, el transporte de los precursores así como las emisiones naturales de COV conduce a una situación de producción neta de O_3 'limitada por NO_x' que se incrementa con la concentración de estos NO_x (Royal Society, 2008). En las zonas montañosas, los aumentos en la concentración de O_3 se relacionan con la altitud debido a condiciones que favorecen las reacciones fotoquímicas, reducen las de eliminación y permiten la entrada de gases desde las capas altas de la troposfera (revisión de Alonso y Bytnerowicz, 2003).

A escala europea, las mayores concentraciones de O_3 se registran en aquellas zonas en las que las condiciones favorecen la formación fotoquímica. Entre ellas destaca el área mediterránea, gracias a los elevados niveles de irradiación y temperatura así como las condiciones de estabilidad atmosférica propios de este clima, especialmente durante los meses de primavera y verano (figura 1.2). Sin embargo, la contaminación por ozono se extiende por amplias zonas en Europa. Por ejemplo, durante el verano del 2004 se superó el umbral de información a la población (180 μg m^{-3}) en más de 2500 ocasiones (Fiala *et al.*, 2003) en comparación con las más de 11000 superaciones que ocurrieron en el verano del 2003 (Fiala *et al.*, 2003), verano en el cual la contaminación por ozono fue muy elevada en toda Europa. Como se puede ver en la figura 1.2, la distribución geográfica de estas superaciones se centra en el sur (Portugal, España, Italia y Grecia) y centro del continente (Francia, Suiza y Alemania).

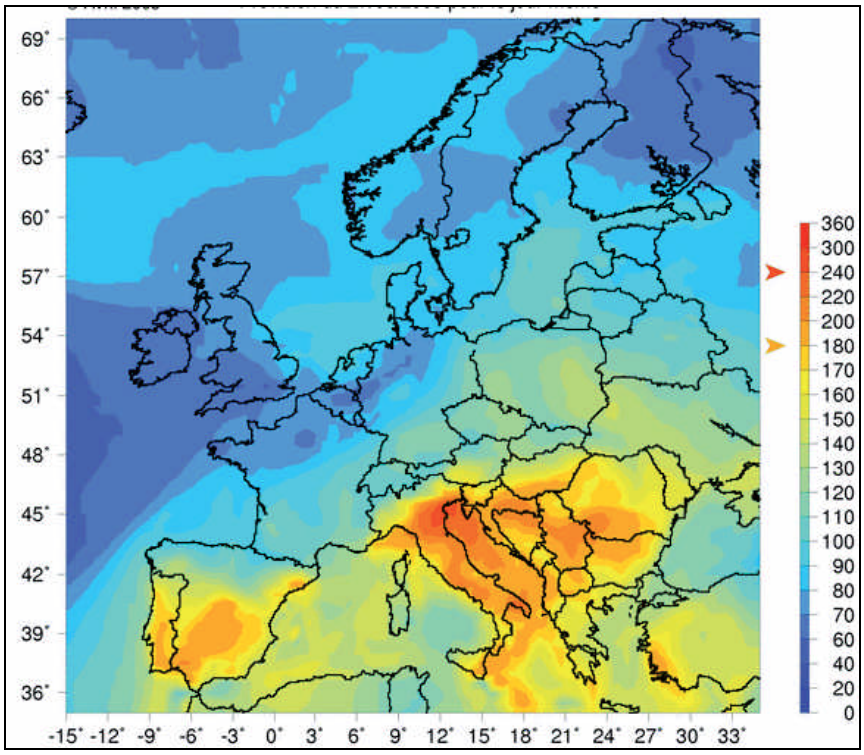

Figura 1.2. Concentración superficial de ozono (26/06/2008) durante el episodio de contaminación registrado en junio del 2008. Adaptada de Gabrielsen *et al.* (2009). (Fuente: *European Topic Centre on Air and Climate Change*. *European Environment Agency*).

2.5. Tendencias en la concentración de ozono troposférico y su relación con el cambio climático

El nivel de fondo actual, 20-45 ppb, no ha permanecido invariable desde que comenzaron las mediciones. Estudios basados en los registros de la concentración de O_3 desde finales del siglo XIX indican un aumento de los niveles de fondo a una tasa de entre el 1 y el 2,5% anual (Vingarzan, 2004; Jonson *et al.*, 2006), asociado al incremento de las emisiones de NO_x, COVs y CO, conocidos precursores del O_3. Este incremento ha sido más acusado en Europa, donde los niveles de fondo en estaciones alejadas de fuentes de precursores se han multiplicado por 5 desde finales del siglo XIX (Marenco *et al.*, 1994) (figura 1.3) y por 2 en los últimos 30 años (Vingarzan, 2004). Existen tendencias similares en zonas montañosas de los Alpes, con incrementos de 0,2 a 0,5 ppb anuales en el periodo 1991-2006 (Ordoñez, 2006), en 13 sitios rurales del Reino Unido, con tasas del orden de 0,14 ppb año^{-1} en el periodo 1990-2006 (Jenkin, 2008), en zonas rurales de la costa oeste de EEUU entre 0,19 y 0,51 ppb año^{-1} entre 1987 y 2004 (Jaffe y Ray, 2007) y en la concentración de fondo hemisférica medida en Mace Head, en la costa atlántica de Irlanda, con una tasa de incremento de 0,3 ppb año^{-1}

entre 1987 y 2007 (Derwent *et al.*, 2008). Estos incrementos se producen en todas las estaciones del año, pero fundamentalmente en invierno y en primavera, aunque los mecanismos que explican tales estacionalidades aún no se han descrito completamente (Jonson *et al.*, 2006).

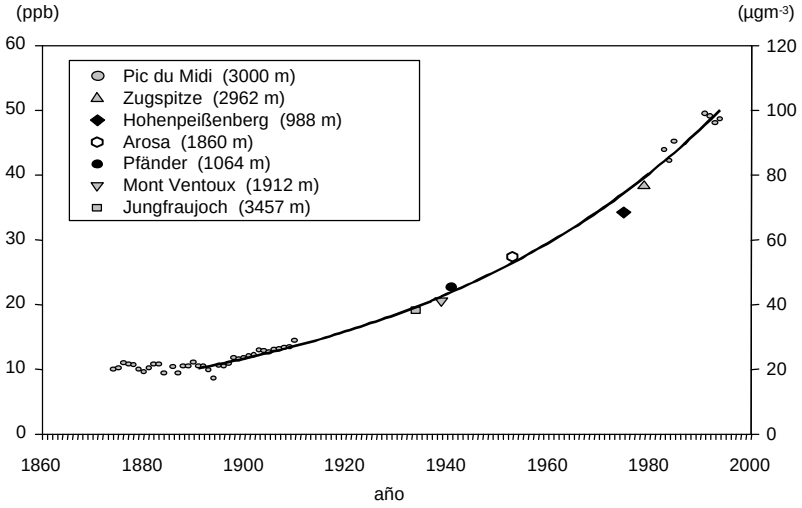

Figura 1.3. Evolución de los niveles de ozono en zonas rurales en Europa (Marenco *et al.*, 1994).

Las tendencias temporales en la concentración de O_3 en aquellas estaciones que no miden niveles de fondo, en cambio, no muestran un panorama uniforme. En muchos lugares de Europa y Norteamérica se ha detectado un descenso en los picos de O_3 troposférico durante los últimos 10 o 15 años (Jonson *et al.*, 2006; Jaffe y Ray, 2007). En el Reino Unido por ejemplo, estos picos han descendido un 30% en el periodo 1986-2001 mientras que la concentración media ha aumentado ligeramente (NEGTAP, 2001), de acuerdo con las tendencias medidas en los niveles de fondo.

Las tendencias de aumento de los niveles de ozono troposférico contrastan con las reducciones en la emisión de precursores que se están produciendo en Europa desde finales de 1980 y los descensos en los niveles de inmisión de NO_2 y COV asociados (Vestreng *et al.*, 2004; Jonson *et al.*, 2006). Esta situación puede ser debida al hecho de que la reducción de emisiones afecta de forma diferente a los sectores considerados en los inventarios de emisiones, lo que se ha traducido en reducciones en la emisión de NO_x menos efectivas en verano que en invierno (Jonson *et al.*, 2006). Los sectores de emisión de precursores relacionados con la producción de energía, industria y plantas de combustión no industrial han liderado el descenso en la emisión de NO_x en Europa,

mientras que el transporte por carretera y otras fuentes móviles han aumentado su contribución (Jonson *et al.*, 2006). En términos de producción de ozono, una menor cantidad de NO_x disponible reduce la inhibición de producción de O_3 bajo condiciones 'limitadas por COV' en invierno, mientras que la producción fotoquímica se ve favorecida en verano con mayor cantidad de NO_x disponible (Jonson *et al.*, 2006). Por otro lado, existen numerosas incertidumbres sobre los efectos de las emisiones de precursores procedentes de otros continentes, de grandes incendios forestales y sobre los efectos del cambio en los patrones generales de circulación atmosférica que puede haber traído consigo el cambio global (Jonson *et al.*, 2006).

La evolución futura de las concentraciones de ozono troposférico debe considerarse en el marco del cambio climático pues la producción fotoquímica de este contaminante está controlada por factores sensibles a las perturbaciones introducidas por el calentamiento global: temperatura, luz solar, la humedad del aire o el transporte de contaminantes (Royal Society, 2008). Además, el propio ozono troposférico se considera como gas invernadero que contribuye al calentamiento global. Sin embargo, la relación entre el cambio climático y la evolución de la concentración de O_3 es compleja. Muchos procesos aún no se conocen con exactitud o están representados de forma muy simple en los modelos fotoquímicos y climáticos que predicen los valores futuros de O_3 (Dentener *et al.*, 2006; Royal Society, 2008). Por ejemplo, el efecto de los niveles de ozono troposférico sobre el sumidero de CO_2 que supone la vegetación en el ciclo del carbono sólo se ha cuantificado recientemente (Sitch *et al.*, 2007). El papel de las emisiones biogénicas de COVs en función de la temperatura, la humedad del suelo o el tipo de vegetación tampoco está descrito en detalle en este tipo de análisis (Sanderson *et al.*, 2003; Royal Society, 2008).

Entre los efectos más destacados del cambio climático que afectan a la producción de O_3 destacan (Royal Society, 2008):

- El aumento de la temperatura mejora la eficiencia de algunas reacciones del ciclo de formación del ozono, aumenta las emisiones biogénicas de COVs y las de NO_x y CH_4 desde los humedales.
- El aumento previsto en la humedad del aire contribuirá al descenso de los niveles de O_3 sobre los océanos. Sobre los continentes, los cambios en la humedad del aire pueden traducirse en incrementos o descensos en función de las condiciones a nivel regional. Por otro lado, el descenso de las precipitaciones afectaría de forma directa aumentando la producción

fotoquímica del ozono y de forma indirecta mediante descensos en la tasa de depósito seco, aumentos en las emisiones de COV o aumentos en la prevalencia de incendios forestales.

- Los cambios previstos en la circulación atmosférica, tanto a nivel global como regional, pueden afectar al transporte transfronterizo de precursores y las condiciones anticiclónicas determinan la existencia de condiciones adecuadas para la producción fotoquímica del ozono.

Las simulaciones de los modelos globales de química y transporte atmosféricos indican que los cambios provocados por el calentamiento de la atmósfera tienden a reducir ligeramente las concentraciones globales de O_3 en 0,8 $\pm$ 0,6 ppb (Dentener *et al.*, 2006). Sin embargo, se espera que estos descensos se produzcan en los lugares poco contaminados (sobre los océanos), mientras que en lugares donde se encuentran las mayores emisiones de precursores, la concentración puede aumentar de forma regional (Dentener *et al.*, 2006). En general, estas simulaciones indican que los escenarios de emisiones antropogénicas son más determinantes que los del cambio climático, provocando aumentos en las concentraciones globales de ozono de entre 1,5 $\pm$ 1,2 ppb y 4,3 $\pm$ 2,2 ppb, según se considere el escenario en el que se cumplen todas las regulaciones legislativas en materia de lucha contra la contaminación del aire o el escenario, más pesimista, en el que se produce un aumento de las emisiones globales de precursores respectivamente (Dentener *et al.*, 2006).

3. EFECTOS DEL OZONO SOBRE LA VEGETACIÓN

Los efectos del ozono sobre las plantas se puede observar en los distintos niveles de organización biológica: bioquímica, fisiología, población y ecosistema. En líneas generales los efectos pueden dividirse en:

- Efectos agudos: producidos por altas concentraciones de ozono durante cortos periodos de tiempo. Generalmente se observan como síntomas visibles, punteaduras de color marrón o pardo-rojizo o bandeados cloróticos sobre las hojas.
- Efectos crónicos: provocados por concentraciones moderadas durante periodos prolongados de tiempo, con incrementos puntuales de la concentración. En estos casos se producen efectos sobre el metabolismo de las plantas que conducen a la senescencia prematura de las hojas, reducciones en el crecimiento y en la productividad.

En los apartados siguientes se revisarán los efectos más importantes a escala de individuo y de ecosistema, con especial atención a los estudios del efecto del ozono sobre los cultivos y sobre las comunidades de pastos.

3.1. Efectos a nivel de planta

El ozono es un gas muy oxidante. Sin embargo, el mecanismo por el cual produce efectos tóxicos sobre la vegetación es muy complejo y aún no se conoce con exactitud. Dos de los procesos más importantes que determinan su toxicidad son la absorción a través de los estomas, la principal vía de entrada al interior de la planta, y la capacidad de los mecanismos de detoxificación y reparación (Massman, 2004; Wieser y Matyssek, 2007). Cuando la concentración de O_3 es lo suficientemente elevada como para superar los mecanismos de defensa de las plantas, se producen efectos directos sobre la fisiología y el crecimiento, aunque concentraciones más moderadas también tienen consecuencias indirectas, debido al gasto energético que supone activar y mantener los procesos de detoxificación y reparación. Cada uno de estos tres aspectos: absorción, detoxificación y efectos se explican en las secciones siguientes de este apartado.

3.1.1. Depósito seco sobre la vegetación

El depósito seco sobre la vegetación resulta de la reacción del O_3 con la superficie de las plantas y de su absorción por parte de las hojas. El O_3 penetra en el interior de los tejidos a través de los estomas, durante el proceso de intercambio gaseoso necesario para la realización de la fotosíntesis. Apenas un 0,01% entra a través de la cutícula de las hojas, aunque puede provocar una degradación de las ceras que la recubren. El flujo de absorción del O_3, al igual que ocurre con otros gases como el CO_2 o el vapor de H_2O, depende de la constante de difusión del gas en el aire, 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 (Thom, 1975; Fowler *et al.*, 1999). La resistencia aerodinámica (R_a) 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. La resistencia de la capa límite (R_b) varía con la velocidad del viento y parámetros morfológicos de la hoja. Determina la concentración de ozono en la capa de aire justo en contacto con la superficie, susceptible de reaccionar con la misma. Finalmente, la fracción de la concentración de O_3 que realmente entra en contacto por la superficie está determinada por la resistencia de superficie (R_c) que presenta dos componentes fundamentalmente, la resistencia cuticular (R_{ext}), que determina la tasa de reacción del ozono 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.

Existe el consenso de que los mayores efectos fitotóxicos del O_3 se producen una vez éste ha sido absorbido por los estomas de las hojas (Fuhrer y Booker, 2003; Massman, 2004; Ashmore, 2005; Fiscus *et al.*, 2005). Por este motivo, se ha prestado especial atención al proceso de absorción estomática del ozono por parte de la vegetación y su relación con los efectos sobre las plantas. 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 (apertura estomática máxima, 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.

Una vez que el ozono ha penetrado en la cavidad subestomática, reacciona rápidamente con las moléculas presentes en el apoplasto (espacio aéreo intercelular, el medio acuoso que rodea las células y los componentes de la pared celular) y en la membrana plasmática. Las concentraciones de ozono registradas en el espacio intercelular son muy bajas en comparación con los niveles de la atmósfera, lo que indica que el ozono se descompone rápidamente tras introducirse por los estomas (Laisk *et al.*, 1989). El O_3 se ve implicado en una serie de reacciones en cadena que producen radicales libres y formas activas del oxígeno, capaces de reaccionar y oxidar multitud de compuestos orgánicos. Estos productos de oxidación interaccionan con los mecanismos antioxidantes de las plantas, que ejercen una función protectora, y desencadenan una serie de procesos dentro de los tejidos vivos que posteriormente se traducen en los efectos que se detectan en la vegetación.

3.1.2. Cuantificación del depósito seco de ozono sobre la vegetación

Dentro del modelo fotoquímico 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 ozono utilizando un 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 se expresa como el múltiplo de la concentración de ozono y el inverso de la resistencia (conductancia) del O_3 para ese paso concreto (ecuación 4). La tasa de depósito se expresa como una velocidad de flujo vertical de depósito en unidades de espacio · tiempo^{-1}.

$$\text{Flujo} = [O_3] \cdot 1/R \qquad (4)$$

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 1.4).

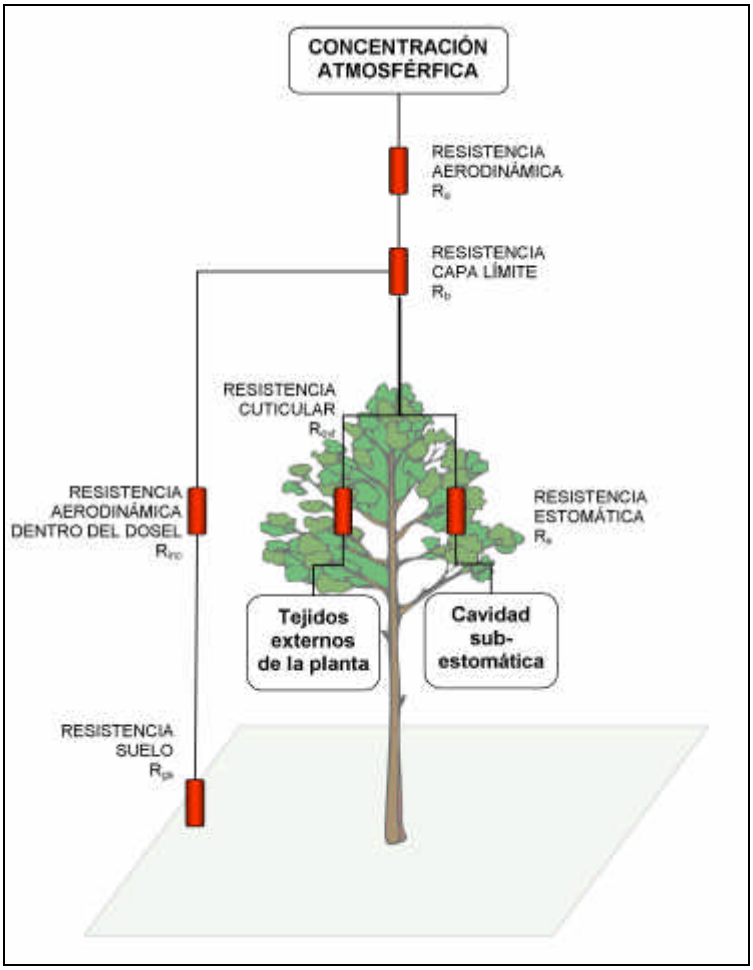

Figura 1.4. Modelo de resistencias para el cálculo del depósito seco de ozono sobre la vegetación. Según la aproximación utilizada por el módulo DO_3SE se simplifican las resistencias del dosel y del suelo. Adaptada de UNECE, 2008.

Dentro del modulo DO_3SE, para modelizar la conductancia estomática se ha seleccionado el modelo multiplicativo, inicialmente propuesto por Jarvis (1976) y modificado por Emberson *et al.* (2000a). El modelo permite el cálculo del flujo de ozono absorbido, susceptible de provocar daños sobre la vegetación (Pleijel *et al.*, 2007). Esta aproximación está basada en la "*big leaf approach*" descrita por Jarvis (1976), según la cual el dosel del cultivo sobre el que se deposita el ozono se representa como una superficie horizontal uniforme. En el modelo multiplicativo la conductancia se calcula como el producto de la conductancia máxima y una serie de parámetros que la corrigen, basados en variables ambientales y particulares de cada especie que actúan de forma independiente (Danielsson *et al.*, 2003). El efecto de esos parámetros se determina utilizando curvas envolventes, líneas que conectan la parte externa de las nubes de puntos en gráficos de conductancia estomática relativa *versus* cada uno de las

variables ambientales (luz, temperatura, etc.) en el momento de la medición. 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). En la ecuación 5 se presenta, como ejemplo de un modelo multiplicativo, el modelo utilizado por DO_3SE (Pleijel *et al.*, 2007; UNECE, 2008).

$$g_s = g_{\max} * [\min(f_{phen}, f_{O3})] * f_{light} * \max[f_{\min}, (f_{temp} * f_{VPD} * f_{SWP})] \qquad (5)$$

Donde g_s es la conductancia estomática (mmol H_2O m^{-2} s^{-1}) y g_{max} es la conductancia estomática máxima. g_{max} 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 el gobierno 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).

3.1.3. Mecanismos de defensa

La formación de radicales libres y de formas activas del oxígeno tiene lugar durante los procesos del metabolismo vegetal normal, por lo que las plantas disponen de sistemas de protección para evitar que se produzcan daños sobre los componentes celulares. En condiciones normales existe un equilibrio entre la formación y la destrucción de estos compuestos, que puede ser alterado por factores ambientales y nutricionales como la sequía, la elevada intensidad de radiación solar, las altas temperaturas o la acción de contaminantes atmosféricos como el ozono.

Un radical libre es cualquier especie química capaz de existir independientemente y que contiene uno o más electrones desapareados, lo que les confiere gran reactividad (Halliwell y Gutteridge, 1989). Los radicales más abundantes en las plantas son aquellos que se derivan del oxígeno, como el radical superóxido (O_2^-) o el ión peróxido (O_2^{2-}), que suele aparecer como peróxido de hidrógeno (H_2O_2) y que a su vez se descompone para formar dos radicales hidroxilo (OH^-). También existen otras formas del oxígeno muy reactivas como el oxígeno singlete que es una forma activada del oxígeno cuando éste absorbe energía.

Los principales sistemas de protección frente a los radicales libres y formas activas del oxígeno son:

- Protección enzimática. Los vegetales presentan una serie de actividades enzimáticas que protegen o retardan la actividad de los radicales oxidantes. Las enzimas superóxido dismutasa, catalasa y peroxidasa catalizan las reacciones de destrucción del superóxido y del H_2O_2. Entre ellas destaca la ascorbato peroxidasa, presente en los cloroplastos, el citoplasma y las mitocondrias. Esta enzima utiliza el ascorbato como sustrato para la reducción del H_2O_2, generándose en la reacción monodehidroascorbato. Esta última molécula puede ser posteriormente regenerada a ascorbato utilizando el poder reductor del NADPH o del glutatión.

- Protección por moléculas antioxidantes. Las células también contienen moléculas capaces de reaccionar directamente con los radicales oxidantes. El ácido ascórbico o vitamina C es un antioxidante que, además de reaccionar directamente con los radicales libres, constituye el sustrato de la enzima ascorbato peroxidasa. El ascorbato está presente de forma habitual en las células y en el apoplasto de múltiples tejidos. Otras moléculas antioxidantes de importancia son el glutatión, que es capaz de reaccionar directamente con el oxígeno singlete y los iones hidroxilo y actúa como cofactor de diversas actividades enzimáticas, y los carotenoides, la vitamina E o las poliaminas. Estas tres últimas ejercen su función antioxidante en medios lípidos.

La fitotoxicidad del ozono está relacionada con la formación de radicales libres y formas activas del oxígeno. Es de esperar por ello que bajo concentraciones elevadas de ozono las plantas activen sus sistemas de protección. 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 como el déficit hídrico (Elvira et al., 1998; Alonso *et al.*, 1999; Alonso *et al.*, 2001). Estas variaciones provocan que la respuesta de la vegetación frente al ozono cambie también en función de las condiciones ambientales, la fenología y el estado fisiológico de la vegetación.

3.1.4. Efectos sobre la fisiología

El ozono provoca cambios sobre la fisiología y el metabolismo de las plantas. Después de interaccionar con los antioxidantes, las primeras moléculas en reaccionar con el ozono 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). Estas reacciones pueden generar, de forma secuencial, efectos sobre la membrana de las células, cambios en el metabolismo y la generación de respuestas frente al estrés, que posteriormente se traducen en disminuciones en las tasas de crecimiento, de productividad, generación de síntomas visibles (clorosis o necrosis) o aceleración de los procesos de senescencia (Heath, 2008), como se verá en los apartados siguientes.

Entre los efectos causados sobre el metabolismo celular 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). En los últimos años se ha observado que el ozono provoca cambios en la expresión de diversos genes (Pell *et al.*, 1997; Heath, 2008). La señal percibida por la célula induce cambios que regulan la transcripción de estos genes. Sin embargo las rutas no son únicas para cada factor de estrés y presentan mecanismos comunes e interacciones en respuesta a diferentes agentes, lo que provoca que el comportamiento del nivel de expresión de los genes de las células expuestas al ozono no parezca ser específico (Schraudner *et al.*, 1997; Heath, 2008).

Los daños provocados por el ozono también influyen sobre la actividad fotosintética, la translocación y el reparto de los asimilados (Andersen, 2003; Fuhrer y Booker, 2003; Ashmore, 2005; Fiscus *et al.*, 2005). En la figura 1.5 se presenta un esquema de los efectos del O_3 sobre la asimilación y el reparto de carbono en las plantas.

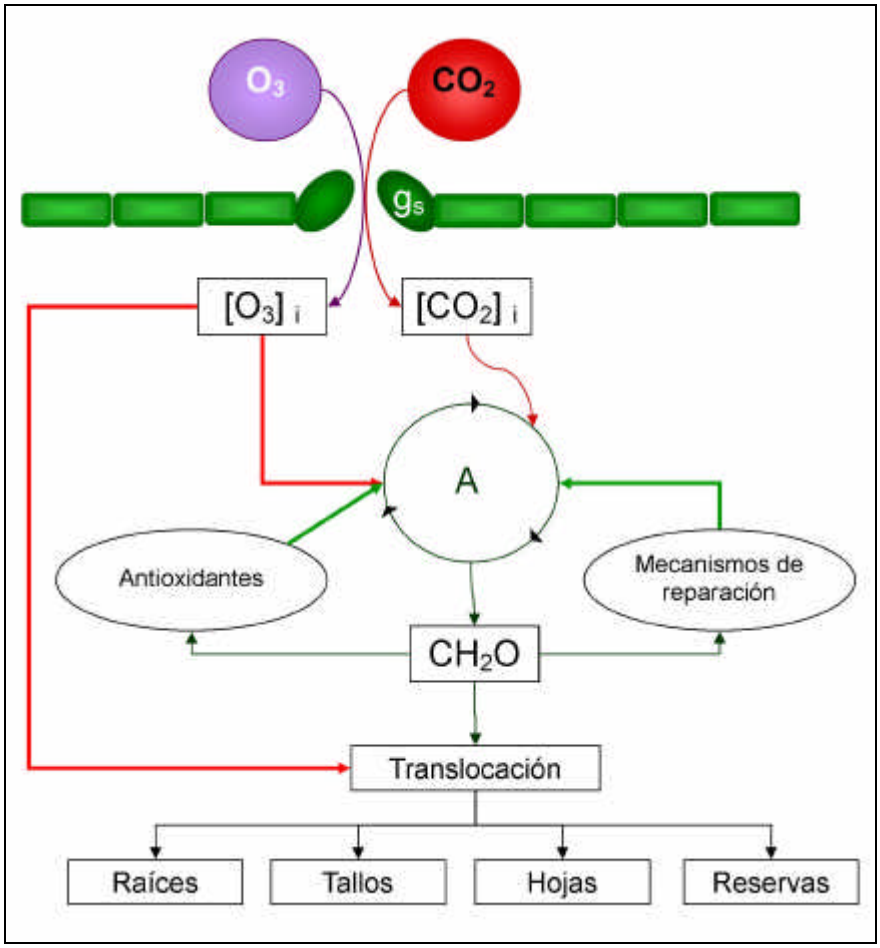

Figura 1.5. Esquema de los efectos del ozono sobre la asimilación y reparto de carbono en las plantas, incluyendo enlaces con los mecanismos de reparación y detoxificación. El dióxido de carbono (CO_2) y el ozono (O_3) penetran al interior de la hoja a través de los estomas, durante el proceso del intercambio de gases. Una vez en la cavidad subestomática, el O_3 reacciona con las moléculas orgánicas del apoplasto y la pared celular y provoca efectos sobre los procesos de asimilación (A) y translocación de los productos asimilados (flechas rojas). Los mecanismos de detoxificación y reparación disminuyen los impactos provocados por el O_3 (líneas verdes), pero suponen un gasto de productos asimilados (CH_2O) que son destinados a las raíces, tallos, hojas y órganos de reserva. g_s, conductancia estomática; $[CO_2]_i$, concentración interna de CO_2; $[O_3]_i$, concentración interna de O_3. (Adaptada de Fuhrer y Booker, 2003).

Numerosos autores han documentado reducciones de la tasa de asimilación que se han relacionado con distintos mecanismos como descensos en la actividad de la Rubisco y del contenido de clorofila, efectos negativos sobre la cadena transportadora de electrones y sobre las células guarda que controlan la apertura estomática, así como aumentos en la tasa de respiración, posiblemente relacionados 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 alterados por el ozono. Entre los mecanismos que se han descrito para explicar los efectos del O_3 sobre la translocación y el reparto de los productos asimilados se encuentran efectos negativos sobre el proceso de carga del floema, aumento de la demanda de asimilados por los sistemas de detoxificación y reparación, que se traducen en aumentos de la tasa de respiración, y alteraciones en el balance del reparto entre las fuentes y los sumideros dentro de la planta, debido a las reducciones de la tasa asimilación y el aumento de la demanda de asimilados en las hojas (McLaughin y McConathy, 1983; Andersen, 2003). Desajustes entre la tasa de fotosíntesis y la translocación de los productos al floema conllevan aumentos en la concentración de azúcares en la hoja, que a su vez disminuyen las tasas de asimilación en algunas especies (Andersen, 2003). En el algodón (*Gossypium barbadense*), exposiciones de corta duración al ozono provocaron aumentos en la concentración de azúcares solubles simultáneamente a descensos en la translocación de asimilados, así como reducciones del flujo de carbono a las raíces comparables a la reducción del flujo de salida de las hojas (Grantz, 2003; Ashmore, 2005).

Las implicaciones de los cambios en el patrón de reparto de asimilados pueden tener consecuencias importantes a largo plazo, pues provocan variaciones en el desarrollo del dosel o las raíces que traen consigo cambios en el acceso a los recursos (nutrientes, agua), en la vitalidad de los bosques, la supervivencia de las plantas herbáceas o provocan cambios en las relaciones de competencia entre especies (Skärby *et al.*, 1998; Ashmore, 2005). Estos patrones de reparto dependen tanto de la actividad de los sumideros como de las tasas de asimilación, por lo que los efectos del O_3 pueden depender de las prioridades del reparto de asimilados en el momento de la exposición al contaminante (Ashmore, 2005). Este hecho es especialmente importante en especies cuya producción de hojas o flores está determinada por su estrategia reproductiva (cereales, gramíneas anuales y otras especies) (Black *et al.*, 2000; Ashmore, 2005). En el caso del trigo (*Triticum aestivum*), se considera el periodo entre la antesis y la maduración del grano como la más sensible en términos de pérdida de producción (Pleijel *et al.*, 1998). En cambio, en otras especies como el tomate (*Lycopersicon esculentum*), el periodo vegetativo resulta ser el más sensible respecto a los efectos

sobre la cosecha, posiblemente debido a los efectos del ozono sobre la producción de biomasa, la razón entre biomasa aérea y subterránea y el retraso en el desarrollo fenológico durante este periodo (Bermejo, 2002). En otros casos, 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, aunque posiblemente sea 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. Numerosos estudios indican que las reducciones del flujo de carbono hacia las raíces provocan cambios en su función hidráulica, lo que afecta a las relaciones hídricas de toda la planta, alteraciones en la absorción de nutrientes del suelo, reducciones en la cantidad de exudados de compuestos orgánicos liberados en el suelo, alteraciones de la fijación de nitrógeno en leguminosas, efectos negativos sobre el desarrollo de las micorrizas, pérdida de vigor de las plantas y cambios en la sensibilidad frente a otros factores de estrés (Skärby *et al.*, 1998; Andersen, 2003; Fuhrer y Booker, 2003; Ashmore, 2005).

3.2. Efectos sobre los cultivos agrícolas

Los efectos del ozono sobre los cultivos agrícolas se centran en tres aspectos: la aparición de síntomas visibles, reducciones sobre la producción y reducciones de la calidad de la cosecha.

Los síntomas visibles más comunes provocados por la fitotoxicidad del O_3 consisten en la aparición de manchas de color negro, rojizo o pardo en el haz de las hojas (Fumagalli *et al.*, 2001; Hayes *et al.*, 2007b) (véase Figura 1.6). Las primeras observaciones de campo sobre daños foliares inducidos por el ozono se registraron sobre la vid (*Vitis vinifera*) a mediados del siglo XX (Richards *et al.*, 1958). Desde entonces se han descrito efectos sobre numerosos cultivos agrícolas y hortícolas. Efectos sobre 22 especies se han documentado tan solo en el área mediterránea, según la revisión de Fumagalli *et al.* (2001), entre las que se encuentran la lechuga (*Lactuca sativa*), el perejil (*Petroselinum sativum*), la achicoria (*Chicorium endiva*) o la espinaca (*Spinacea oleracea*), en las que la presencia de síntomas visibles sobre las hojas determina su valor comercial (Velissariou, 1999). Los síntomas visibles se han identificado sobre extensas zonas agrícolas, como el levante español, utilizando sandía

(*Citrullus lanatus*) como especie indicadora en una zona de 600 por 40 Km. (Gimeno *et al.*, 1995). En este sentido, dentro del ámbito europeo se han desarrollado numerosos experimentos de biomonitorización. Estos experimentos, que han utilizado varias especies como judía (*Phaseolus vulgaris*), trébol blanco (*Trifolium repens*), trébol subterráneo (*Trifolium subterraneum*), soja (*Glycine max*) sandía, tomate y tabaco (*Nicotiana tabacum*), han demostrado que la aparición de síntomas visibles cubre extensas áreas repartidas por toda Europa (Benton *et al.*, 2000; Hayes *et al.*, 2007b). Sin embargo, la aparición de síntomas visibles no siempre se traduce en efectos negativos sobre la producción y viceversa (Black *et al.*, 2000), aunque pueden ser muy importantes en aquellos cultivos cuyo valor comercial se base en el aspecto de las hojas.

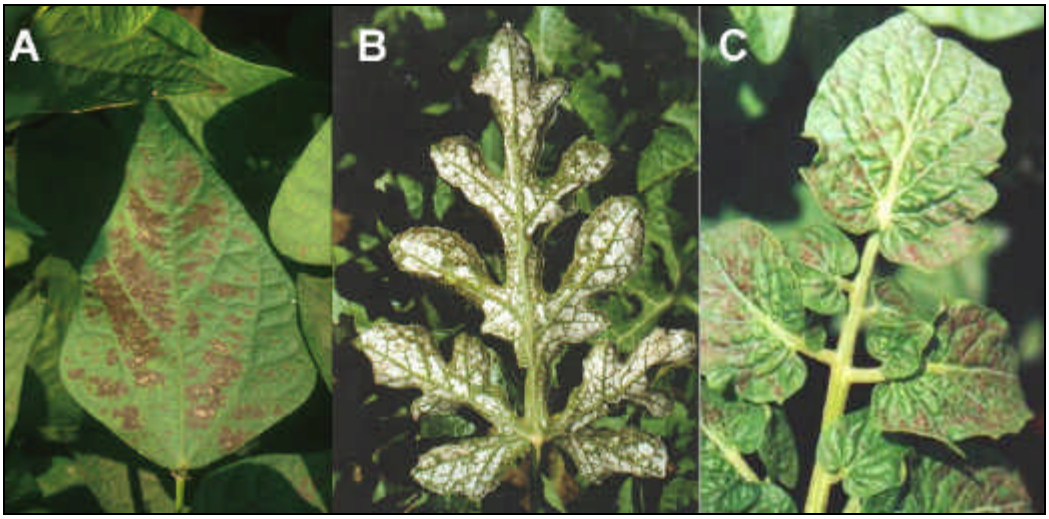

Figura 1.6. Fotos de síntomas visibles causados por el O_3 en hojas de (A) judía (*Phaseolus vulgaris*), (B) sandía (*Citrullus lanatus*) y (C) tomate (*Lycopersicon esculentum*) identificados durante experimentos de fumigación en cámaras de techo descubierto.

Existe una abundante cantidad de estudios que indican que las concentraciones actuales de ozono son lo suficientemente altas como para provocar efectos negativos sobre la productividad de los cultivos agrícolas (Black *et al.*, 2000; Fumagalli *et al.*, 2001; Ashmore, 2005; Feng y Kobayashi, 2009), aunque estos impactos dependen de la concentración, la duración y el periodo en que ocurre la exposición en relación con el estado de desarrollo de la planta (Pleijel *et al.*, 1998; Black *et al.*, 2000; Soja *et al.*, 2000).

En numerosos trabajos en los que se han expuesto al ozono tanto los órganos vegetativos como los reproductivos de las plantas, los efectos encontrados se han asociado a la senescencia prematura de las hojas, descensos en la interceptación de la luz, la concentración de clorofila y la actividad fotosintética, con su consiguiente disminución en la disponibilidad de fotosintatos, y la alteración del reparto del carbono fijado (Black *et al.*, 2000; Fuhrer y Booker, 2003; Fiscus *et al.*, 2005). Los efectos del ozono sobre las estructuras reproductivas de las plantas se han asociado por tanto a efectos indirectos sobre los órganos vegetativos y posteriormente sobre la cantidad y el reparto de productos asimilados hacia las estructuras reproductivas (Black *et al.*, 2000). Sin embargo, otros estudios también han identificado efectos directos del ozono sobre las estructuras reproductivas (Black *et al.*, 2000). Entre otros procesos, se han encontrado retrasos en la iniciación del periodo de floración, descensos de la producción de flores, de la tasa de germinación de las semillas, así como reducciones en la producción de semillas y frutas en numerosas especies agrícolas y hortícolas entre las que se encuentran especies de alto valor como *Prunus* (almendro, melocotón, albaricoque), tabaco, tomate, maíz (*Zea mays*), colza (*Brassica napus*), trigo (*Triticum aestivum*), trigo duro (*Triticum durum*), arroz (*Oryza sativum*), soja, judía, sandía y patata (*Solanum tuberosum*) (Gimeno *et al.*, 1999; Black *et al.*, 2000; Pleijel *et al.*, 2002; Bermejo, 2002; De la Torre, 2004; Feng y Kobayasi 2009). Mills *et al.* (2007), por ejemplo, identificaron en su análisis de las relaciones entre la producción (peso) y la exposición al ozono en 19 cultivos agrícolas y hortícolas procedentes de numerosos experimentos, hasta 17 especies afectadas de las cuales 9 fueron calificadas como sensibles y 8 como moderadamente sensibles.

Otro aspecto a tomar en consideración sobre los efectos del ozono en la productividad de los cultivos consiste en los retrasos en la maduración de las cosechas, como los registrados en sandía o tomate (Gimeno *et al.*, 1999; Bermejo, 2002). Bajo concentraciones medias de ozono de 47 ppb (7 h dia^{-1}) se redujo la producción de tomate (en términos de peso) en la cosecha temprana respecto a la cosecha control (en la que el ozono se elimina del aire mediante filtros); posteriormente la cosecha tardía compensó estas pérdidas de forma que el efecto global resultó nulo (Bermejo, 2002). De igual manera, el ozono afectó de forma más importante la cosecha temprana de sandía acentuando las pérdidas provocadas por la reducción de la producción (Gimeno *et al.*, 1999).

Además de la gran cantidad de efectos y especies afectadas descritos en los trabajos mencionados, se ha encontrado también una elevada variabilidad en la sensibilidad frente al ozono, no sólo entre especies si no también entre variedades de una misma especie (Black *et al.*, 2000; Biswas *et al.*, 2008; Feng *et al.*, 2008). Se han registrado variaciones de los efectos del O_3 sobre la producción de trigo expuesto a una concentración promedio de 40 ppb (7 h dia^{-1}) de entre el 6 y el 14% en un meta-análisis (Feng y Kobayashi, 2009), mientras Heagle *et al.* (1988) apunta diferencias de hasta un 40% en la respuesta de la producción de 5 variedades de trigo frente al O_3 y de un 30% comparando 13 variedades de soja.

Varios estudios se han centrado, precisamente, en la evolución de la sensibilidad de distintas variedades de cultivos como el trigo. Estos estudios indican que, entre las variedades de trigo analizadas, las modernas son más sensibles al ozono que las más antiguas (Barnes *et al.*, 1990; Velissariou *et al.*, 1992; Barnes *et al.*, 1999; Plejiel *et al.*, 2006). Este hecho está posiblemente en relación, al menos de forma parcial, con la selección de variedades que presenten tasas de intercambio gaseoso más elevadas en las variedades modernas, característica que contribuiría a aumentar su productividad (Velissariou *et al.*, 1992), pero que también permite una mayor entrada del ozono hacia el interior de la planta (Pleijel *et al.*, 2006). La selección directa de variedades de cultivos resistentes al ozono se ha realizado con éxito en varias especies (Barnes *et al.*, 1999). De igual manera se han identificado variedades sensibles a este contaminante, que han permitido, por comparación entre variedades sensibles y resistentes, el establecimiento de sistemas de biomonitorización como ocurre con el tabaco (Heggestad, 1991), el trébol blanco (Heagle *et al.*, 1994) o la judía (Burkey *et al.*, 2005). El sistema del trébol blanco, por ejemplo, se basa en la comparación de las pérdidas de biomasa causadas por el ozono en dos variedades, resistente y sensible, de trébol bajo un protocolo experimental estándar y se ha utilizado ampliamente en Europa para indicar riesgos potenciales de daños producidos por el O_3 sobre la vegetación (Hayes *et al.*, 2007b).

El estudio de los efectos del ozono sobre la calidad de la cosecha ha recibido menor atención que los efectos sobre la producción, aunque se ha reconocido su importancia (Fuhrer y Booker, 2003). En el caso del trigo, se han descrito incrementos en la concentración y cambios en la composición de las proteínas en el grano así como incrementos en la actividad de la enzima a-amilasa (Pleijel *et al.*, 1999; Piikki *et al.*,

2008b). Cambios en estos parámetros pueden afectar al uso final que se da a la cosecha y por lo tanto al precio que el grano alcanza en el mercado (Lawlor y Mitchell, 2001). También se han encontrado descensos en el contenido en azúcares en frutos de sandía (Gimeno *et al.*, 1999) y tomate (Bermejo, 2002), cultivos hortícolas de gran importancia en el levante español. Al igual que ocurrió con las pérdidas de producción, las cosechas tempranas de tomate, que alcanzan un mayor valor comercial, resultaron ser las más afectadas en cuanto a descensos en el contenido de azúcares y tamaño del fruto, importantes parámetros que determinan la calidad en este cultivo (Bermejo, 2002). Por lo tanto las pérdidas de calidad pueden agravar las consecuencias de las pérdidas de producción provocadas por el O_3.

Otros cultivos en los que se han evaluado los efectos del ozono sobre la calidad son la colza (Bosac *et al.*, 1998), con descensos en el contenido de aceite, proteínas y carbohidratos en la semilla; la vid (Soja *et al.*, 2004), donde la cantidad de carbohidratos destinados a las uvas se redujo con la exposición; la soja (Heagle *et al.*, 1998), que mostró ser moderadamente tolerante al ozono en los parámetros evaluados; y la patata (Vorne *et al.*, 2002) especie en la que la exposición al ozono mejoró la calidad de la cosecha.

3.3. Efectos sobre los pastizales

Al igual que ocurre con los cultivos, el ozono también provoca síntomas visibles, reducciones del crecimiento y la producción de semillas y descensos en la calidad nutritiva de las especies que componen los pastos.

Varios estudios documentan la aparición de síntomas visibles e incrementos en la senescencia sobre especies de pastos después de ser expuestas a cantidades de O_3, en algunos casos, relativamente bajas en comparación con los índices establecidos para la protección de la vegetación (Nebel y Fuhrer, 1994; Davison y Barnes, 1998; Bergmann *et al.*, 1999; Benton *et al.*, 2000; Bermejo *et al.*, 2003; Hayes *et al.*, 2007a) (véase apartado 4.2). Estos resultados evidencian de nuevo la existencia de una elevada variabilidad en la sensibilidad de las especies, lo que ha llevado a desarrollar clasificaciones basadas en la aparición de síntomas visibles, en las que las leguminosas han mostrado ser más susceptibles al ozono respecto a otras familias como las gramíneas (Davison y Barnes, 1998; Bermejo *et al.*, 2003). Sin embargo, la posición de

las especies en estas clasificaciones puede sufrir variaciones en función de los parámetros de interés (aparición de síntomas visibles, reducciones en la biomasa aérea o subterránea, etc.), pues la presencia de síntomas visibles no implica necesariamente la aparición de impactos sobre la biomasa o sobre el ecosistema (Reiling y Davison, 1992; Davison y Barnes, 1998; Bergmann *et al.*, 1999; Bermejo *et al.*, 2003; Gimeno *et al.*, 2004a; Bender *et al.*, 2006).

Numerosos trabajos han analizado la sensibilidad al ozono en términos de crecimiento en más de 171 especies de pastos europeos y estadounidenses, provenientes de diferentes comunidades vegetales (véase tabla 1.1). Sin embargo este número resulta escaso en comparación con las aproximadamente 7500 especies herbáceas descritas sólo en Europa y tomando en consideración la importancia de los pastos, que cubren alrededor del 50% de la superficie de este continente (Ashmore *et al.*, 2007).

Procedencia de las especies	**nº Spp**	**Efectos considerados**	**País**	**Referencia**
Prado productivo	2	Biomasa aérea Composición Interacción con SO_2	EE.UU.	Kohut *et al.*, 1988
Prado productivo	2	Biomasa aérea Composición Interacción con disponibilidad de H_2O	EE.UU.	Rebbeck *et al.*, 1988; Heagle *et al.*, 1989
Pasto establecido del sur del R.U.	7	Biomasa aérea Composición	R.U.	Evans y Ashmore, 1992
Pastos subalpinas	16	Biomasa Interacción con temperatura	Noruega	Mortensen y Nilsen, 1992
Naturales del R.U.	32	Biomasa aérea Razón biomasa aérea/subterránea	R.U.	Reiling y Davison, 1992
Pasto zonas calcáreas	10	Biomasa aérea Composición	R.U.	Ashmore *et al.*, 1995
Pasto creado artificialmente	4	Biomasa aérea Composición Interacción con siega	R.U.	Ashmore y Ainsworth, 1995
Naturales de Alemania	12	Biomasa aérea	Alemania	Bergmann *et al.*, 1995
Pasto productivo	2	Biomasa aérea Composición	Suiza	Nussbaum *et al.*, 1995
Pasto zonas calcáreas del R.U y Europa	5	Biomasa aérea Biomasa subterránea Razón biomasa aérea/subterránea Longitud de la raíz	R.U.	Warwick y Taylor, 1995
Naturales de Reino Unido	38	Biomasa aérea	R.U.	Ashmore *et al.*, 1996
Naturales de Alemania	6	Biomasa aérea	Alemania	Bergmann *et al.*, 1996

Naturales del R.U.	1	Biomasa aérea Interacción con frío	R.U.	Foot *et al.*, 1996
Prado productivo	3	Biomasa aérea Composición	Suecia	Pleijel *et al.*, 1996
Naturales de Suecia	27	Biomasa aérea	Suecia	Pleijel y Danielsson, 1997
Pasto nativo de Dinamarca	8	Biomasa aérea	Dinamarca	Mortensen, 1997
Pasto de sucesión de bosque	40	Cobertura vegetal Diversidad	EE.UU.	Barbo *et al.*, 1998
Naturales de Suiza	24	Biomasa aérea	Suiza	Bungener *et al.*, 1999
Naturales de Suecia	2	Biomasa aérea Producción de flores	Suecia	Danielsson *et al.*, 1999
Humedal	10	Biomasa aérea Biomasa subterránea Razón biomasa aérea/subterránea	Holanda	Franzaring *et al.*, 2000
Pradera natural	3	Biomasa aérea Composición Interacción con disponibilidad de H_2O	Suiza	Nussbaum *et al.*, 2000
Cenagal	12	Biomasa aérea Biomasa subterránea Razón biomasa aérea/subterránea	R.U.	Power y Ashmore, 2002
Dehesa	19	Biomasa aérea Biomasa subterránea Razón biomasa aérea/subterránea Dimensiones del dosel Producción de flores y semillas Interacción con competencia interespecífica y depósito de nitrógeno Calidad nutritiva	España	Gimeno *et al.*, 2004a,b Sanz *et al.*, 2005, 2007
Naturales de Holanda	4	Biomasa aérea Interacción con competencia interespecífica	Holanda	Tonneijck *et al.*, 2004
Zonas agrícolas	25	Biomasa aérea Producción de flores y semillas Germinación de semillas	Alemania	Bender *et al.*, 2006
Pradera natural zonas elevadas	33	Biomasa aérea	R.U.	Hayes *et al.*, 2006
Pradera natural zonas elevadas	10	Biomasa aérea Composición Interacción con disponibilidad de nutrientes y competencia	R.U.	Samuelsson *et al.*, 2006
Pradera nórdica seca	7	Biomasa aérea Composición Interacción con CO_2	Finlandia	Rämö *et al.*, 2006
Pasto establecido de zonas calcáreas	38	Composición	R.U.	Twaites *et al.*, 2006
Pasto pre-alpino	53	Biomasa aérea Composición	Suiza	Volk *et al.*, 2003, 2006
TOTALES	171		11	

Tabla 1.1. Resumen de estudios sobre la sensibilidad del crecimiento y otros parámetros de las especies de pastos frente al ozono.

Estos estudios muestran una elevada variabilidad interespecífica en la respuesta encontrada, con especies sensibles (reducen su biomasa aérea o subterránea), tolerantes e incluso estimuladas tras la fumigación con ozono. Parte de esta variabilidad puede deberse a las diferentes estrategias de crecimiento adoptadas por cada especie, que provocan variaciones en los periodos de máxima sensibilidad al ozono, diferencias en la capacidad de detoxificación o en la morfología de la hoja (Davison y Barnes, 1998; Bender *et al.*, 2006; Bassin *et al.*, 2007). Ciertas especies anuales de la familia de las leguminosas, por ejemplo, han mostrado ser especialmente sensibles durante el periodo vegetativo; exposiciones de 2 meses de duración provocaron reducciones de la biomasa aérea del 70% (Gimeno *et al.*, 2004a). En cambio la especie perenne *Centaurea jacea* mostró mayor sensibilidad durante el periodo reproductivo (Bassin *et al.*, 2004), mientras que *Plantago major*, también perenne, lo hizo durante la fase de plántula (Lyons y Barnes, 1998). Estas diferencias se pueden traducir en cambios en la composición del pasto debidos al ozono, causados por la variabilidad de la sensibilidad de sus componentes. Los experimentos con mezclas de especies suelen indicar una reducción de la proporción de leguminosas frente a las gramíneas, generalmente más resistentes, aunque la magnitud de estos cambios está sujeta, nuevamente, a muchos factores que modulan la respuesta (Davison y Barnes, 1998; Twaites *et al.*, 2006; Bassin *et al.*, 2007).

La variabilidad entre especies en la respuesta frente al ozono también se produce entre individuos de la misma especie. Varios estudios han demostrado la existencia de una variabilidad intraespecífica, en la que diferentes poblaciones de plantas o genotipos muestran una respuesta diferente frente a la exposición al ozono (por ejemplo Danielsson *et al.*, 1999; Bassin *et al.*, 2004). La composición de los pastos es el resultado de la selección de los genotipos mejor adaptados a las condiciones locales, en las que el ozono es un factor más (Bassin *et al.*, 2007). Así se ha observado que la exposición al ozono puede cambiar la composición de una población mediante la selección de los genotipos más resistentes en los lugares más contaminados, lo que incrementa su resistencia comparada con otras poblaciones procedentes de lugares menos expuestos (Lyons *et al.*, 1997; Bassin *et al.*, 2004). Este hecho puede amortiguar la respuesta de las comunidades de pastos frente al ozono, en comparación con los experimentos que tratan con un solo genotipo, aunque en el largo plazo, la pérdida de

los genotipos más sensibles trae como resultado una pérdida de la biodiversidad genética (Bassin *et al.*, 2007).

Otros efectos del ozono detectadas en especies de pastos se dan sobre la reproducción. Los efectos negativos del ozono sobre la producción de flores y semillas, la viabilidad, longevidad, latencia y la germinación de las semillas así como sobre el vigor de las semillas producidas puede tener efectos potencialmente serios para la supervivencia y el establecimiento de la progenie, principalmente durante esta última fase, aunque se encuentran poco estudiados para las especies de pastos (Black *et al.*, 2000; Gimeno *et al.*, 2004b).

Se ha reconocido que los resultados de algunos de los experimentos de sensibilidad pueden haber sobreestimado los efectos respecto a las condiciones de campo. Variables como la radiación fotosintética activa, la humedad del suelo y del aire, la disponibilidad de nutrientes en el suelo o las relaciones de competencia entre especies pueden modular la respuesta de las especies frente al ozono respecto al óptimo representado en dichos resultados (Bassin *et al.*, 2007). Los experimentos con mezclas de especies y comunidades intactas de plantas indican que la respuesta global no es el resultado de la suma de las respuestas individuales de cada especie, sino que existen multitud de factores que la modulan como la diversidad de especies, la presencia de genotipos con diferente sensibilidad al ozono, las relaciones de competencia, la estructura del dosel o las técnicas de manejo (Bassin *et al.*, 2007), lo que puede retardar la aparición de efectos. Un experimento a largo plazo sobre una comunidad de pastos pre-alpinos en Suiza (Volk *et al.*, 2006) mostró reducciones del crecimiento superiores al 20%, en comparación con el control, después de 5 años de exposición a niveles elevados de ozono generados mediante un sistema de fumigación al aire libre. Sin embargo, los cambios en el crecimiento no resultaron significativos hasta el cuarto año del experimento, lo que muestra un retardo en la aparición de efectos frente a los experimentos desarrollados en cámaras de fumigación con comunidades más jóvenes.

Como resultado de los cambios inducidos por el ozono en la química foliar (cambios en la concentración de minerales, proteínas y carbohidratos y en la composición de la pared celular de las hojas), diversos estudios han puesto de manifiesto la pérdida de la calidad nutritiva del pasto para los rumiantes en términos de consumo y digestibilidad (Krupa *et al.*, 2004; Muntifering *et al.*, 2006). Se han registrado aumentos en la concentración de fibras y de ligninas que reducen el valor

nutritivo de las hojas de leguminosas del género *Trifolium*, componentes importantes debido a su importancia como recurso alimenticio para los herbívoros (Sanz *et al.*, 2005; Muntifering *et al.*, 2006). Estos cambios se han asociado a una aceleración de la senescencia de las hojas (Muntifering *et al.*, 2000; Sanz *et al.*, 2005), un efecto del ozono bien descrito para otras especies como el trigo (Ojanpera *et al.*, 1992; Gelang *et al.*, 2000; Pleijel *et al.*, 2007).

Los efectos inducidos por el ozono a nivel de individuo o población tienen su reflejo en los procesos que ocurren a nivel de ecosistema, que se revisan en la sección siguiente.

3.4. Efectos a nivel de ecosistema

Los efectos del ozono a largo plazo sobre los ecosistemas resultan aún desconocidos en su mayoría, por ejemplo los que se refieren a la modificación de los ciclos biogeoquímicos como los del carbono y el nitrógeno (Ashmore, 2005). Experimentos con mesocosmos indican que el ozono puede influir sobre los microorganismos del suelo mediante cambios en la química de la hojarasca y las raíces o de los exudados de la raíz (Olszyk *et al.*, 2001; Andersen, 2003). Esto puede traducirse en reducciones de las tasas de descomposición de la materia orgánica muerta y cambios en la química y la microbiología del suelo (Andersen, 2003; Fuhrer y Booker, 2003) que a su vez, puede afectar a la respuesta de las plantas frente al O_3 incrementando o reduciendo su sensibilidad (Davison y Barnes, 1998). Además, los cambios en las relaciones de competencia interespecíficas pueden conllevar reducciones en la proporción de leguminosas, más sensibles al ozono, que se registran en mezclas artificiales (véase revisión de Bassin *et al.*, 2007), con efectos potencialmente negativos sobre la fijación del nitrógeno atmosférico (Andersen, 2003) y sobre la calidad nutritiva del pasto para los animales herbívoros (Sanz *et al.*, 2005; Muntifering *et al.*, 2006).

Sin embargo, los experimentos con comunidades intactas de pastos perennes muestran un panorama complejo en cuanto a variaciones en la composición de especies, observados a largo plazo y en el cual resulta difícil determinar el papel del O_3 (Ashmore, 2005; Twaites *et al.*, 2006; Bassin *et al.*, 2007). Por otro lado, en los pastos dominados por terófitas anuales, como los de las dehesas de la península Ibérica, la composición de la comunidad está determinada por el banco de semillas del suelo (Peco *et al.*, 1998).

Por este motivo el aporte anual de semillas, afectado por el ozono en al menos tres especies de trébol, resulta crucial para la supervivencia de esta vegetación (Gimeno *et al.*, 2004b).

El O_3 actúa también como un factor abiótico adicional en los bosques que predispone a los árboles frente a otros tipos de estrés, fundamentalmente la sequía, el ataque de patógenos y las deficiencias nutricionales (Skärby *et al.*, 1998). La presencia de otros gases también puede provocar interacciones con los efectos del O_3. Existen evidencias que prueban que niveles elevados de CO_2, como los previstos por escenarios de cambio global, reducen la absorción de O_3 al reducir la apertura estomática de las hojas (Fiscus *et al.*, 1997); además aumenta la disponibilidad de productos asimilados para su utilización en procesos de defensa y detoxificación frente al O_3 (Heagle *et al.*, 1999). Por todo ello, el CO_2 podría ejercer una función protectora frente al O_3. Sin embargo, los experimentos con O_3 y CO_2 muestran una elevada variabilidad en la respuesta, para la cuál aún resulta necesaria más investigación (Fiscus *et al.*, 2005).

Los estudios más completos sobre los efectos del ozono sobre los ecosistemas se han realizado en las montañas de San Bernardino, cerca de Los Ángeles (EE.UU.) (Miller y McBride, 1999). Históricamente, esta zona estaba dominada por bosques mixtos de coníferas de *Pinus ponderosa* y *Pinus jeffreyi* debido a su resistencia a los incendios forestales. Sin embargo, ambas especies son sensibles al ozono, y muestran síntomas visibles, reducciones en la longevidad de las acículas, en el crecimiento y en la producción de semillas cuando están expuestos a este contaminante, así como una mayor sensibilidad frente al ataque de patógenos. La pérdida de competitividad de estas especies frente a otras más tolerantes y la exclusión del fuego propició un cambio en la composición de especies, modulado por una interacción compleja de factores donde se encuentran el depósito de nitrógeno, el régimen de precipitaciones y el manejo del bosque (Arbaugh *et al.*, 2003). Otro estudio (Jones *et al.*, 2004) mostró que el ataque de escarabajos de la corteza se agudizó, tras cuatro años consecutivos de sequía, en aquellos árboles que mostraban síntomas visibles causados por el ozono en zonas con altos niveles de depósito de nitrógeno.

De forma similar a los bosques de las montañas de San Bernardino, también se han documentado empeoramientos en la salud de los bosques que se localizan en el área Mediterránea europea (Bussotti y Ferretti, 1998) y de los bosques de coníferas que circundan la ciudad de México (Fenn *et al.*, 2002).

4. EVALUACIÓN DE LOS EFECTOS DEL OZONO SOBRE LA VEGETACIÓN

4.1. El Convenio de Ginebra

La necesidad de establecer un programa conjunto a nivel internacional para desarrollar políticas y estrategias de gestión medio ambiental que controlen la contaminación atmosférica fue el origen del Convenio de Ginebra sobre Transporte a Larga Distancia y Transfronterizo de Contaminantes Atmosféricos (*Convention on Long-Range Transboundary Air Pollution*, CLRTAP UNECE). El Convenio de Ginebra (CLRTAP) es la organización encargada de generar los protocolos sobre reducción de emisiones de contaminantes que deben ser reflejados en cada país mediante decretos específicos. Entre los objetivos y compromisos adquiridos por los países firmantes del Convenio 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. El Convenio de Ginebra 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 31 países, entre ellos los países miembros de la Unión Europea y por tanto España.

Dentro del organigrama del Convenio de Ginebra se encuentra el Grupo de Trabajo sobre Efectos (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. Las actividades del WGE se llevan a cabo mediante el establecimiento de Programas de Cooperación Internacional (ICPs). La evaluación y control de los efectos de la contaminación atmosférica sobre la vegetación se llevan a cabo en el ICP *Vegetation* (cultivos agrícolas y hortícolas y vegetación herbácea), ICP *Forests* (bosques) y el ICP *Integrated Monitoring*.

El Convenio de Ginebra 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 (UNECE, 1988). 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 (CIAM, 2007)

Los niveles críticos establecidos para los distintos contaminantes y receptores se consensúan 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, como se verá en el apartado 4.3.

4.2. Índices para la evaluación del riesgo de daños por ozono y su relación con la respuesta de los receptores: establecimiento de niveles críticos

Un elemento fundamental en el estudio de los efectos de los contaminantes atmosféricos consiste en la cuantificación de la relación entre la exposición al contaminante y la respuesta biológica. La determinación de funciones exposición – respuesta requieren el empleo de índices de exposición al contaminante que definan lo más adecuadamente posible las características de ésta que tienen mayores repercusiones para la planta, ya sean concentraciones medias, concentraciones por encima de un umbral, niveles máximos, etc. Estas relaciones tienen un gran interés en la determinación de los umbrales de daño de los contaminantes sobre receptores específicos, lo que tiene implicaciones en el establecimiento de guías de calidad del aire y el establecimiento de normativas legales. Cuanto más acertado sea el índice de exposición seleccionado, mejor será el resultado de las funciones exposición – respuesta y la efectividad de los umbrales de protección.

Diversos programas de investigación en EE.UU. y Europa, NCLAN (*National Crop Loss Assessment Network*), EOTCP (*European Open-Top Chamber Programme*) e ICP-*Vegetation*, han tenido como objetivo el establecimiento de las relaciones causa efecto de los receptores vegetales frente al O_3. Durante el desarrollo de estos estudios se han utilizado diferentes índices y establecido umbrales para la protección de los receptores de mayor sensibilidad que han tenido reflejo en la legislación para el control

de la contaminación por ozono. 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 (Pleijel *et al.*, 2002):

- 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 (AF_{st}).

Los índices de exposición basados en promedios, establecidos en 1988, se basan en el cálculo de medias de concentración del contaminante en el aire durante periodos de tiempo determinados (una hora, 10, 12 ó 24 horas). El programa NCLAN, por ejemplo, estableció como índice el promedio estacional de 7 y 12 horas para describir las pérdidas de productividad provocadas por el ozono sobre cultivos agrícolas (Heagle *et al.*, 1987), mientras que la legislación europea contemplaba promedios horarios y diarios para la protección de la vegetación (Directiva 92/72/CEE). El problema más importante de este tipo de índices es que no distinguen entre las diferentes distribuciones de concentración, ya que picos notables de contaminación en 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 (Lefohn, 1994) cuando la realidad puede ser diferente. Por este motivo, los índices desarrollados con posterioridad a los promedios consideran la exposición acumulada durante el ciclo de vida de la planta.

Los índices de exposición basados en la concentración acumulada se desarrollaron como respuesta a los problemas planteados por los índices basados en promedios. En Europa, los datos experimentales de pérdidas de productividad del trigo por causa del O_3 obtenidos en el programa EOTC mostraron una gran correlación con la exposición acumulada en horas diurnas por encima de determinados umbrales (30 y 40 ppb) durante el ciclo de vida de la planta (véase figura 1.7). La terminología europea para este tipo de índices es AOT (*Accumulated exposure Over a given Threshold*, exposición acumulada de ozono por encima de un umbral determinado). La definición de los niveles críticos basados en la exposición acumulada se formuló por primera vez en 1996 (Kärenlampi y Skärby, 1996; Fuhrer *et al.*, 1997), utilizando el AOT40, la

exposición acumulada por encima de 40 ppb durante horas diurnas. El umbral utilizado, 40 ppb, no es en realidad un umbral de efecto si no que se fundamenta en la penalización de las concentraciones por encima del nivel de fondo en el hemisferio norte.

El AOT40, acumulado durante 3 ó 6 meses según el tipo de vegetación, es el índice contemplado actualmente en la legislación europea para la protección de la vegetación (Directiva 2008/50/CE; véase apartado 4.3). Además se han desarrollado numerosos experimentos en los que se relaciona el AOT40 con los efectos del ozono sobre multitud de especies. Una revisión de estas relaciones para especies agrícolas puede encontrarse en Mills *et al.* (2007).

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 que pueden modular la toxicidad del ozono, ya que las concentraciones más elevadas pueden no estar asociadas con los mayores flujos de entrada al interior de la planta, donde el O_3 tiene sus efectos más dañinos (Fuhrer *et al.*, 1997). Desde 2002 (Karlsson *et al.*, 2003) se acordó considerar el establecimiento de niveles críticos basados en la dosis absorbida del contaminante para aquellos tipos de vegetación para los que exista un conocimiento científico suficiente.

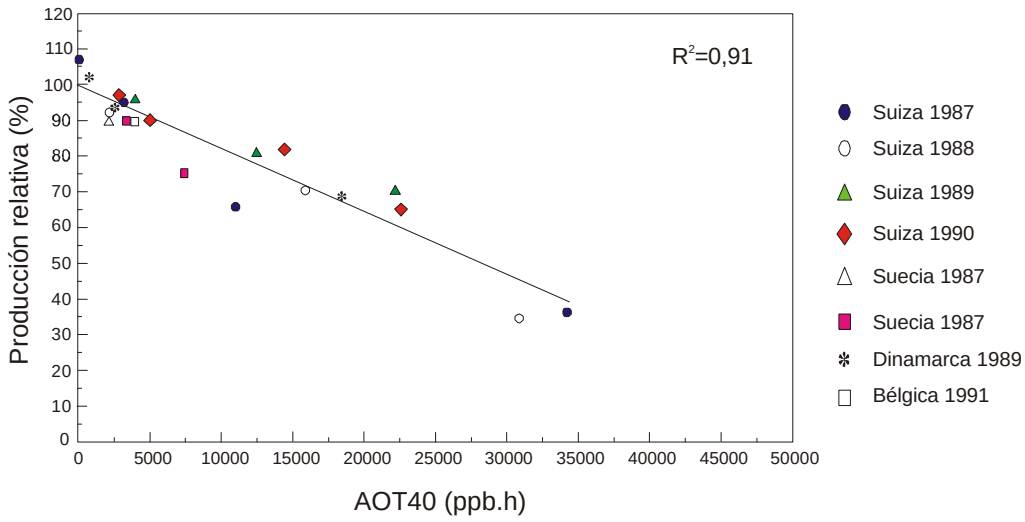

Figura 1.7. Relación exposición – respuesta para trigo basado en el índice de exposición AOT40 (exposición acumulada por encima de 40 ppb durante tres meses en horas diurnas). Los datos empleados en esta función proceden de la base datos europea recopilada durante el programa EOTC (adaptada de Fuhrer y Ashmore, 1996).

La absorción del ozono por la planta está gobernada por varios factores que, como ya se ha comentado, controlan la apertura de los estomas, como son la radiación solar, la humedad del aire y del suelo, la temperatura y el estado de desarrollo de la planta o fenología. Según estas consideraciones, los niveles críticos basados en la dosis absorbida consideran no solo la concentración de ozono en el aire, si no la cantidad de ese ozono que es realmente absorbido por las plantas, modulada por diferentes factores como las condiciones microclimáticas en las que el cultivo se desarrolla. Estos factores pueden reducir las pérdidas esperables, calculadas a partir del índice AOT40.

La determinación de este último tipo de niveles críticos, se basa en el establecimento de relaciones dosis-respuesta en las que la dosis se define como la cantidad acumulada de contaminante absorbido por encima de un umbral determinado por unidad de tiempo y superfice, abreviado como $AF_{st}Y$ (mmol m^{-2}) (A*ccumulated ozone stomatal Flux over a threshold of Y nmol* O_3 m^{-2} s^{-1}, flujo estomático de ozono acumulado sobre un umbral de *Y* nmol O_3 m^{-2} s^{-1}).

Con el fin de calcular los índices $AF_{st}Y$, es necesario conocer el comportamiento estomático de los receptores del contaminante y las tasas de depósito del O_3 sobre las superficies, como se ha comentado en el apartado 3.1.2. En la actualidad existen relaciones provisionales para el trigo, la patata, trébol blanco, haya y abedul basadas en esta aproximación (Mills *et al.*, 2003; UNECE Mapping Manual 2007; Pleijel *et al.*, 2007) mientras que para otras especies como vid, tomate, girasol, maíz, remolacha, raigrás perenne, encina, pino de Alepo o coscoja aún están en desarrollo (Emberson *et al.*, 2005; Ashmore *et al.*, 2007; Elvira et al., 2007; Alonso *et al.*, 2008). Existe también la necesidad de validar los modelos adoptados para la predicción de la conductancia estomática en condiciones de campo y demostrar que los índices basados en la absorción de ozono proporcionan relaciones más consistentes con las pérdidas de producción o reducción del crecimiento que los índices basados en la concentración. Sin embargo, aún existen incertidumbres respecto al cálculo de la dosis absorbida, especialmente relacionados con la inclusión de los mecanismos de detoxificación y defensa de las plantas que pueden modular la toxicidad de la dosis absorbida de O_3 y a la aplicabilidad de esta aproximación para establecer los niveles de protección para la vegetación como iniciativa legislativa.

La metodología seguida en este trabajo va encaminada hacia la determinación de relaciones dosis – respuesta basadas en esta última aproximación, y puntualizar

incertidumbres que se plantean en la extrapolación de los cálculos de riesgo basados en la dosis absorbida de ozono a escala regional, nacional y europea.

4.3. Legislación europea sobre ozono troposférico

En la Tabla 1.2 se indican los valores umbrales que no deben sobrepasarse para la protección de la vegetación y la salud humana indicados en la Directiva Europea de Calidad del Aire (2008/50/EC). La Tabla 1.3 presenta los umbrales (niveles críticos) establecidos en el Convenio de Ginebra para la protección de cultivos, especies herbáceas y forestales (UNECE, 2008).

El índice de exposición a O_3 AOT40, se emplea tanto en la Directiva como en el Convenio para la definición de los umbrales de protección para la vegetación. De acuerdo con la Directiva, para cumplir con el objetivo a corto plazo (a cumplir en el 2010), el índice 3-meses (mayo-julio) AOT40 no debe superar el valor de 9.000 ppb.h (18.000 µg m^{-3} h) como media de 5 años. Como objetivo a largo plazo, sin fecha de cumplimiento delimitada, la misma Directiva indica el valor del índice 3-meses AOT40 de 3.000 ppb.h (6.000 µg m^{-3} h) que no debe superarse para asegurar la protección de la vegetación. El Convenio de Ginebra señala distintos niveles críticos para O_3 en función del tipo de vegetación: un valor del índice AOT40 acumulado durante 3 meses de 3.000 ppb.h (6.000 µg m^{-3} h) para cultivos y especies herbáceas y un valor de 5.000 ppb.h (10.000 µg m^{-3} h) durante 6 meses para especies leñosas acumulados durante la estación de crecimiento.

En el caso de la protección de la salud humana, la Directiva Europea utiliza como índice de exposición a O_3 la media máxima diaria de 8 horas. Para cumplir con el objetivo definido a corto plazo (2010), la media máxima diaria de 8 horas no debe superar en más de 25 días al año el valor de 61 ppb (120 µg m^{-3}) (media de 3 años); un valor que no debería superarse en ningún caso para cumplir con el objetivo fijado a largo plazo (sin fecha delimitada de cumplimiento).

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 que se encuentra en revisión en la actualidad (CIAM, 2007).

Directiva Europea de la Calidad del Aire 2008/50/EC
Valores Objetivo (a cumplir el 1 de enero de 2010)
Protección de la Vegetación: *El índice AOT40 acumulado durante 3 meses (mayo-julio) debe estar por debajo de 18.000 µg m^{-3} (9.000 ppb h) (media de 5 años)*
Protección de la Salud Humana: *La media diaria máxima de 8 h no podrá superar 120 µg m^{-3} (61 ppb) más de 25 días al año (media de 3 años)*
Objetivos a largo plazo
Protección de la Vegetación: *El índice AOT40 acumulado durante 3 meses (mayo-julio) debe estar por debajo de 6.000 µg m^{-3} (3.000 ppb h)*
Protección de la Salud Humana: *La media diaria máxima de 8 h no podrá superar 120 µg m^{-3} (61 ppb)*

Tabla 1.2. Valores objetivo y objetivos para el ozono troposférico para la protección de la vegetación y la salud humana recogidos en el Anexo VII de la Directiva 2008/50/CE de Calidad del Aire.

Vegetación	Nivel crítico	Periodo de tiempo	Efecto
Cultivos	Cultivos agrícolas: AOT40 3.000 ppb h Horticolas: AOT40 6.000 ppb h	Cultivos agrícolas: 3 meses Hortícolas: 3,5 meses	Reducción de la producción (5%)
Vegetación semi-natural (herbácea)	AOT40 3.000 ppb h	3 meses (o ciclo vital si es más corto)	*Perennes*: reducción crecimiento *Anuales*: Reducción de crecimiento y/o semillas (10%)
Árboles	AOT40 5.000 ppb h	Estación de crecimiento	Reducción crecimiento (5%)

Tabla 1.3. Niveles críticos para la protección de la vegetación (3 tipos, cultivos, vegetación herbácea y árboles) recogidos por el CLRTAP de la UNECE. Se especifica también el periodo de acumulación y el efecto en que se basa el nivel crítico. La superación del límite solo indica riesgo de daño.

5. OBJETIVOS DE ESTA TESIS

Los trabajos presentados en esta tesis se integran en el marco de las actividades del ICP-*Vegetation* (*Internacional Cooperative Programme on Effects of Air Pollution on Natural Vegetation and Crops*) del CLRTAP. Una de las prioridades del programa ICP-*Vegetation* consiste en desarrollar las metodologías de modelización de los flujos estomáticos de ozono para realizar evaluaciones del riesgo de daños, así como el cálculo de relaciones dosis – respuesta que permitan establecer niveles críticos para la protección de los cultivos y las especies herbáceas. Respecto a estas líneas de investigación, los objetivos generales de la presente memoria, que posteriormente se concretan en cada uno de los capítulos, son:

1. Ampliar el número de especies agrícolas y hortícolas, de importancia económica en Europa, para las que se dispone de relaciones de dosis – respuesta. Las especies consideradas son la lechuga y el trigo de invierno. Este objetivo general comprendía a su vez los siguientes:
    a. Desarrollo de modelos de conductancia estomática de estas dos especies.
    b. Análisis de los efectos del ozono sobre distintos aspectos de la producción.
    c. Desarrollo de funciones dosis – respuesta.
2. Explorar la consideración de los parámetros de calidad de la producción en el desarrollo de relaciones de dosis – respuesta para especies agrícolas (trigo) y herbáceas de pastos productivos (mezclas de raigrás y trébol).
3. Contribuir al debate sobre el establecimiento de los niveles críticos de ozono basados en el flujo estomático acumulado mediante su comparación con el índice utilizado en la actualidad, AOT40, basado en la exposición.
4. Estudiar las implicaciones de la variabilidad intra- e interespecífica de la fisiología y la sensibilidad al ozono en el establecimiento de relaciones de dosis – respuesta.

5. Analizar las interacciones del efecto del ozono con otros factores como la nutrición del suelo y la competencia entre especies en comunidades de pastos productivos.
6. Desarrollar nuevas metodologías para el cálculo de flujos estomáticos de ozono para comunidades de vegetación semi-natural: pastos de dehesa. Este objetivo incluye:
    a. Explorar la interrelación entre la humedad del suelo, el índice de área foliar y el periodo de crecimiento de los pastos anuales de dehesas.
    b. Acoplar la variación interanual de la dinámica del pasto con el flujo estomático de ozono.
    c. Estudiar la variabilidad interespecífica en parámetros de intercambio gaseoso.

El texto de esta memoria se organiza en cuatro capítulos, en cada uno de los cuales se describe un experimento individual que explora diferentes aspectos de los objetivos anteriormente mencionados. Allí se especifican los objetivos, metodología, resultados y conclusiones obtenidos en cada caso. Estos capítulos van seguidos de una discusión general y finalmente unas conclusiones, en las que se ofrece una visión de conjunto en relación con el cálculo de flujos estomáticos de O_3 y el desarrollo de relaciones dosis-respuesta.

2. ESTABLECIMIENTO DE RELACIONES DOSIS-RESPUESTA DE OZONO PARA LA LECHUGA: UN CULTIVO HORTÍCOLA CLAVE

Goumenaki E, Gonzalez Fernández I, Papanikolaou A, Papadopoulou D, Askianakis C, Kouvarakis G, Barnes J. (2007). Derivation of ozone flux-yield relationships for lettuce: A key horticultural crop. Environmental Pollution 146: 699-706.

Resumen

En este trabajo se establecen relaciones de dosis-respuesta para cultivos de lechuga. Con este objetivo se ha desarrollado un modelo multiplicativo de conductancia estomática (g_s), según el cual se puede describir la influencia que determinados parámetros ambientales ejercen sobre g_s. El modelo fue calibrado utilizando una base de datos de medidas de g_s tomadas en campo durante un experimento desarrollado en Creta (Grecia). De forma paralela se realizó un experimento de fumigación con ozono en cámaras descubiertas para determinar el efecto del ozono sobre la producción de la lechuga. Ambos experimentos se realizaron respetando las prácticas agronómicas normales para este cultivo.

El análisis de valores de g_s modelizados frente a medidos mostró que el modelo multiplicativo explicó un 51% ($P < 0,001$) de la variabilidad observada en las medidas.

También se realizó una comparación entre índices basados en la concentración de ozono e índices basados en el flujo absorbido.

Se encontraron regresiones significativas entre la dosis de ozono absorbida y la producción cuando se emplearon umbrales inferiores a los 4 nmol m^{-2} s^{-1}. Estos análisis mostraron que las relaciones dosis-respuesta más importantes se dieron cuando la dosis se acumulaba por encima de valores umbrales muy bajos o nulos. En estos casos, las relaciones dosis-respuesta explicaron hasta un 80% ($P < 0,05$) de la variabilidad encontrada en la respuesta de la lechuga frente al ozono en términos de producción.

Available online at www.sciencedirect.com
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Environmental Pollution 146 (2007) 699–706
ENVIRONMENTAL POLLUTION
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Derivation of ozone flux-yield relationships for lettuce: A key horticultural crop

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Received 22 December 2005; received in revised form 7 June 2006; accepted 6 August 2006

Establishment of ozone flux-yield relationships for a commercially-important horticultural crop grown widely in the Mediterranean.

Abstract

Ozone flux-response relationships were derived for lettuce, employing a multiplicative approach to model the manner in which stomatal conductance is influenced by key environmental variables, using a dataset collected during field experimentation in Crete and yield-response relationships derived from parallel open-top chamber experiments. Regional agronomic practices were adopted throughout. Computed *versus* measured data revealed that the derived model explained 51% ($P < 0.001$) of the observed variation in stomatal conductance. Concentration-based indices were compared with flux-based indices. Analyses revealed a significant relationship between accumulated stomatal ozone flux and yield employing flux threshold cut-offs up to 4 nmol m^{-2} s^{-1}. Regressions employing very low or zero flux thresholds resulted in the strongest yield-flux relationships (explaining $\approx 80\%$ ($P < 0.05$) of the variation in the dataset).

Keywords: Lettuce; Stomatal conductance; $AF_{st}Y$; AOT40; Critical flux *versus* exposure

1. Introduction

Current levels of ozone (O_3) pollution are known to be high enough to depress crop yield and drive subtle shifts in the diversity of wild plant communities in many parts of Europe, North America and Asia (Davison and Barnes, 1998; Barnes et al., 1999a,b; Emberson et al., 2001; Ashmore, 2005; Huixiang et al., 2005). The problem is recognised, and the United Nations Economic Commission for Europe (UNECE), Convention on Long-Range Trans boundary Air Pollution (CLRTAP) was established in 1983 with a remit to strive toward pan-European risk assessment and initiate policies to curb emissions of pollutants, and their precursors, that constitute a significant regional (i.e. trans-national) pollution problem. An effects-orientated approach was adopted to establish pollutant abatement policies (UNECE, 1988). Initially, this was based on the identification of the critical exposure to specific pollutants i.e. the concentration of the pollutant in the atmosphere above which direct adverse effects on receptors (such as humans, plants, ecosystems or materials) may occur (Bull, 1991). This concentration-based approach was not dissimilar in principal to other exposure-based indices employed across the world as the basis for pollution control policy (see

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doi:10.1016/j.envpol.2006.08.009

Fuhrer et al., 1997), the most useful of which integrate exposure over time and provide greater weight to higher concentrations (see Musselman et al., 1994; Heagle et al., 1995; Chevone et al., 1998; Heagle and Stefanski, 2000; Grünhage et al., 2001). The adoption of accumulated exposure to hourly ozone concentrations over 40 ppb during daylight hours over the 3 months of the growing season that the target crop is active in the field – an index more commonly referred to as the AOT40 – constituted the basis of this approach and allowed mapping of the risks posed by ozone to crops across Europe (UNECE, 1994). The limitations associated with such exposure-based (i.e. concentration-based) indices, rather than absorbed (or preferably, effective) dose, are recognized and it is accepted that the AOT40 approach results in the overestimation of impacts in many circumstances, because environmental and plant-specific variables influencing O_3 uptake are not considered (Fuhrer et al., 1997). As a consequence, considerable recent attention has focused on the development of models to estimate stomatal ozone flux, with current approaches focusing on the generation of multiplicative algorithms reflecting the manner in which stomatal conductance is influenced by key environmental (particularly temperature, VPD, PAR), physical (particularly soil moisture deficit) and inherent considerations (e.g. leaf ontogeny and circadian controls) (Jarvis, 1976; Emberson et al., 2000), as well as accounting for atmospheric and boundary layer conductance (Fowler et al., 2001; Grünhage et al., 2000; Gerosa et al., 2005). Possibly the major limitation associated with the current approaches to modeling ozone flux is that it is not currently possible to estimate the 'effective' dose of the pollutant, only the absorbed dose, due to a lack of 'simple' formulae facilitating the prediction of shifts in ozone detoxification and damage repair capacity driven by climate and other variables (Massman et al., 2000; Plochl et al., 2000).

To date, attempts to map the risks posed to vegetation by ground-level ozone pollution have understandably focussed on wheat and potato grown under Northern European conditions – as these represent the only major datasets available (Danielsson et al., 2003; Pleijel et al., 2004). Relatively few datasets exist that can be used to derive ozone-flux response relationships for key Mediterranean crops considered 'at risk' (e.g. high value field-grown horticultural crops such as tomatoes, lettuce, spring onions and soft fruit). Given the paucity of data, the present study was instigated in a bid to establish ozone flux-response relationships for lettuce (*Lactuca sativa* L.), a crop generally classified as sensitive to ozone (UNECE, 2004) and of particular significance in Southern Europe, a region responsible for 65% of trans-European lettuce production (amounting to 3.2 10^6 Mt per annum; FAOSTAT, 2005). Stomatal conductance measurements were made during a field experiment conducted in Crete; a Mediterranean island located on the South-Eastern fringe of Europe which is subject (for the majority of the year) to air masses heavily influenced by human activity in Central and Eastern Europe (Kouvarakis et al., 2000). Ozone pollution is a recognised problem in the region (Barnes et al., 1990; Velissariou et al., 1992; Fumagali et al., 2001), but insufficient monitoring and response data are available to facilitate an assessment of the risks posed to natural and managed vegetation – a familiar situation in the Mediterranean.

2. Materials and methods

2.1. Plant culture and fumigation

2.1.1. Field studies – Crete

Seedlings of lettuce (*Lactuca sativa* L. cv. Paris Island) were propagated in a glasshouse in Crete in springtime employing standard commercial practices. Once the fourth true leaf had begun to emerge (17th March 2004), 200 plants were transferred to a site (latitude 35°20′N, longitude 25°8′E, 10 m.a.s.l.) in the suburbs of Heraklion, Crete, and transplanted into a pre-prepared field (using a spacing of 25 cm between plants) in double rows 50 cm apart at 100 cm intervals. Plants were subject to standard agronomic management practices in the region, employing a range of agrochemical treatments (but avoiding specific chemicals known to affect plant responses to ozone). Air temperature (T, °C), relative humidity (RH, %), photosynthetically active radiation (PAR, μmol m^{-2} s^{-1}) and soil moisture content (SM, %), were recorded using cross-checked Skye sensors linked to a 16-channel datalogger (Skye Instruments Ltd, Powys, UK). Soil moisture content was determined using a capacitance sensor (Decagon Devices Inc., model EC-10). Ozone concentration (nmol mol^{-1}) at canopy height was measured with the aid of a photometric analyser (Thermo Electron Instruments model 49C, Franklin MA, USA) serviced at weekly intervals and calibrated against primary and secondary standards by a much-experienced team at the University of Crete (led by Prof. N. Mihalopoulos, Dept. of Chemistry, University of Crete). Measurements of stomatal conductance to water vapour (g_{H2O}) were recorded over the lifespan of the sixth leaf using a Li-Cor LI-1600 steady state porometer (Li-Cor, Lincoln, NE, USA). The experimental period coincided with the commercial growing season for lettuce in Greece. Measurements were taken every 1 or 2 days, with the exception of holiday periods (5th–15th April and 29th April–2nd May). On measurement days, g_{H20} was generally measured for *c.* 4 h. On four occasions near the beginning of the experiment (31st March, 2nd April, 5th April and 3rd May) g_{H2O} was tracked over the course of the day for 8 to 9 h (from 11:00 until dusk at 19:00 h). No measurements were taken early in the morning, so as to avoid artefacts associated with early-morning dew formation/water microfilms on leaf surfaces.

2.1.2. Field studies – U.K.

Open-top chamber experiments were performed at Newcastle University's Field Station at Close House, Heddon-on-the-Wall, Northumberland, in Northern England (latitude 54°59′N, longitude 1°48′W, elevation 30 m.a.s.l.). Seedlings (*Lactuca sativa* L. cvs. Paris Island and Salad Bowl) were raised in controlled environment chambers ventilated with charcoal/Purafil®-filtered air (CFA) as described elsewhere (Zheng et al., 1998). When the fourth true leaf began to emerge, plants were individually transferred to pots containing 5 dm^3 of Levington F2 compost. Five pots of each cultivar were randomly assigned to each of sixteen open-top chambers. All open-top chambers were ventilated with particulate-filtered ambient air (NFA). As of July 1st, ozone was injected into the ductwork ventilating twelve of the sixteen chambers (with ozone treatments randomly assigned to chambers) to achieve four targeted daytime treatments (7 h d^{-1}; 10:00–17:00): NFA, NFA + 25 nmol mol^{-1} O_3, NFA + 50 nmol mol^{-1} O_3, NFA + 75 nmol mol^{-1} O_3). Open-top chambers were of the rigid type (i.e. constructed from angular aluminium, clad with standard horticultural glass), incorporating a plenum at a height of 2.3 m to reduce ambient air incursion at higher wind speeds. Each OTC was ventilated (day and night) *via* a perforated annulus positioned 1.3 m above ground-level using a dedicated fan supplying sufficient NFA to achieve 2 air changes min^{-1} in each chamber. Environmental data were gathered at plant height both inside and outside one of the OTCs using a combination of cross-calibrated Skye PAR, temperature and RH sensors, linked to a 16-channel logging unit (Skye Instruments Ltd, Powys, UK) and a portable logger (Intelisys, Manchester, UK). Ozone levels were monitored at plant height using a PC-controlled multi-line sampling system (see Barnes et al., 1995) linked to a photometric analyser (Dasibi 1008PC supplied by SupportingU Ltd., Aylesbury, Bucks.,

UK) serviced at weekly intervals and calibrated monthly against an instrument in-turn calibrated against a secondary standard every 6 months. Plants were harvested at commercial maturity (on August 22nd) after 7.5 weeks' exposure to controlled levels of ozone.

2.1.3. Laboratory-based controlled environment studies

Seeds of a 'Cos' lettuce (*Lactuca sativa* L., cv. Paris Island) were sown in 3.5 × 3.5 cm plugs of 38 × 24 × 6.5 cm modules containing Levington F2 compost (Scotts Company Ltd., Ipswich, UK). These were placed in a controlled environment chamber ventilated with charcoal/Purafil®-filtered air, and then – once the fourth true leaf had begun to emerge (after three weeks) – plants were individually transplanted into pots containing 1.5 dm^3 of the same compost. Ten plants of each variety were then placed in four fully randomized blocks inside each of six pre-programmable controlled environment chambers described in detail elsewhere (Zheng et al., 1998). Duplicate chambers were ventilated with charcoal/Purafil®-filtered air (CFA; <5 nmol mol^{-1} O_3), CFA plus 75 nmol mol^{-1} O_3 for 8 h (08:00–16:00) or CFA plus 100 nmol mol^{-1} O_3 for 8 h d^{-1} (08:00–16:00). Chambers were illuminated by metal-halide floodlights (Siemens, HR400H housing fitted with 400 W HQI-T lamps, Osram, St. Helens, Merseyside, UK) providing a PPFD of approximately 400 μmol m^{-2} s^{-1} at canopy height, delivered as a 14 h photoperiod (07:00–21:00). Air temperature in the chambers ranged from a day-time maximum of 25 °C to a night-time minimum of 14 °C. Plants were watered as required, and were supplied with a commercial nutrient solution (20-20-20, Chempak Products, Essex, UK) once per week.

Stomatal conductance to water vapour (g_{H2O}) was recorded every two hours over a diel cycle after 28 d exposure. Measurements were made *in situ* with a Delta-T Devices AP-4 porometer (Delta-T Devices, Cambridge, UK) on the abaxial surface of the youngest fully-expanded leaf of six plants per variety and treatment, alternating between plants exposed to CFA or CFA plus O_3. Rates of CO_2/H_2O exchange were recorded on the sixth leaf on six independent plants exposed to CFA or CFA plus 100 nmol mol^{-1} O_3 [8 h d^{-1}]. Measurements were made *in situ* using an automated Parkinson leaf cuvette (model AUTO-PLC-B, PP Systems, Hitchin, UK) linked to an infrared gas analyzer (IRGA, model Ciras II, PP Systems, Hitchin, UK) employing a PPFD of 400 μmol m^{-2} s^{-1}, a cuvette atmospheric CO_2 concentration of 360 ± 5 μmol CO_2 mol^{-1} dry air and a leaf temperature of 22 ± 0.1 °C (i.e. close to the conditions inside plant growth chambers). Measurements were made between 14:00 and 16:00 h and spanned the period from just before the stage of full leaf expansion to senescence. Rates of g_{H2O} were calculated according to Von Caemmerer and Farquhar (1981).

2.2. Concentration-based indices

Cumulative ozone exposure i.e. AOT20, 30, 40 and 50 (the accumulated exposure to ozone over a threshold of 20, 30, 40 and 50 ppb, respectively, during daylight hours) was calculated according to UNECE (2004). Modified AOT indices (mAOT) were calculated, considering PAR and vapour pressure deficit (VPD), as performed by Karlsson et al. (2004) for short-term critical levels.

2.3. Derivation of multiplicative stomatal conductance model

A database comprising 483 measurements of abaxial surface stomatal conductance to water vapour (g_{H2O}) recorded over a range of conditions over the life-span of the 6th leaf was used to parameterise a multiplicative, Jarvis-style 'big leaf' model (Jarvis, 1976), employing the approach adopted by Emberson et al. (2000). Values for maximum (g_{max}) and minimum (g_{min}) conductance per unit leaf area were calculated from the average of the data above the 90th percentile and the average below the 10th percentile, respectively. Boundary line-derivation of model parameters used to simulate stomatal conductance is shown in Fig. 1. Boundary lines were fitted using an objective approach, selecting relevant points at the outer margins of the data cloud pursuant with recommendations in the latest edition of the Mapping Manual (UNECE, 2004). Boundary lines fitted using this approach showed close correspondence with non-linear regression fits employing the 99th percentiles extracted from the dataset and ranked in 20 groups of homogeneous width. All boundary line parameters employed for modelling stomatal conductance fell within the 95% confidence intervals determined by non-linear regression.

Stomatal conductance (g_{H2O} [mmol m^{-2} s^{-1}]) was simulated using a multiplicative algorithm of the following formulation:

$$g_{H2O} = \max\{(g_{max} * g_T * g_{PAR} * g_{VPD} * g_{SM} * g_{phen} * g_{AF_{st0}} * g_{time}); (g_{max} * g_{min})\} \quad (1)$$

Where g_{max} represents the maximum stomatal conductance and the impacts of the remainder of the parameters in the model are expressed in terms of their relative effect on g_{max}, scaled from 0 (g_{H2O} equal to g_{min}) to 1 (g_{H2O} equal to g_{max}). The effect of temperature, photosynthetically active radiation, vapour pressure deficit (VPD, kPa), soil moisture and time of day on g_{H2O} are represented by g_T, g_{PAR}, g_{VPD}, g_{SM} and g_{time}, accordingly. g_{phen} and g_{AFst0} represent the manner in which relative stomatal conductance is influenced by leaf ontogeny (measured as growing degree days (GDD, °C days) and cumulative ozone flux ($AF_{st}0$, mmol m^{-2}), respectively.

Flux calculations employed hourly mean ozone concentrations, with g_{H20} converted to g_{O3} using the difference in the diffusivity coefficient between ozone and water (in air), and took into account cuticular resistance. Ozone uptake per unit leaf area (F_{st}, nmol m^{-2} s^{-1}), was estimated as described in the UNECE Mapping Manual (2004) using Eq. (2). Stomatal conductance was derived by applying Equation 1 under conditions that were continuously monitored. A correction factor, derived from extensive controlled environment studies on the same cultivar of lettuce (see Fig. 1h), was employed to weight ozone effects on stomatal conductance at ozone concentrations exceeding those employed for the formulation of the model, as the dataset revealed that high ozone concentrations promoted changes in stomatal behaviour. The difference in stomatal conductance between CFA and ozone treatments was linearized, and the ratio applied to the daylight average ozone concentration recorded during OTC experimentation.

$$F_{st} = c_{O3} * g_{H2O} * D_{H2O/O3} * r_c * (r_b + r_c)^{-1} \quad (2)$$

where, F_{st} represents ozone uptake per unit leaf area (nmol m^{-2} s^{-1}), c_{O3} is the hourly average ozone concentration (nmol m^{-3}), g_{H2O} is the stomatal conductance to water vapour (m s^{-1}), $D_{H2O/O3}$ represents the difference in diffusivity between ozone and water in air (0.613) (Nobel, 1983), r_c (s m^{-1}) is leaf surface resistance ($(g_{H2O} + g_{ext})^{-1}$), where g_{ext} represents leaf cuticular conductance – employing a value of 1/2500 sensu UNECE (2004), r_b represents quasi-laminar resistance $(1.3 * 150 * [L * u(z_1)^{-1}]^{1/2}$ (s m^{-1}) where $u(z_1)$ represents wind speed at height z and L (m) is the cross-wind leaf dimension. Wind speed measurements were used to calculate quasi-laminar resistance in the field experiments and a value of 5 m s^{-1} was employed in OTC experiments.

Accumulated hourly stomatal fluxes (mmol m^{-2}) above a threshold (Y, nmol m^{-2} s^{-1}) were calculated:

$$AF_{st}\,Y = \sum (F_{st} - Y) * 3600 * 10^{-6} \quad (3)$$

2.4. Statistical analysis

Data were first tested for normality of distribution and homogeneous variance using Kolmogorov–Smirnov or Shapiro–Wilks and Levene tests, prior to examining the impacts of ozone on biomass using one-way and two-way ANOVA. The level of significance was determined using the least significant difference calculated at the 5% level. Linear regression analyses were used to evaluate the strength of the relationships between yield and a variety of ozone indices. All statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, USA).

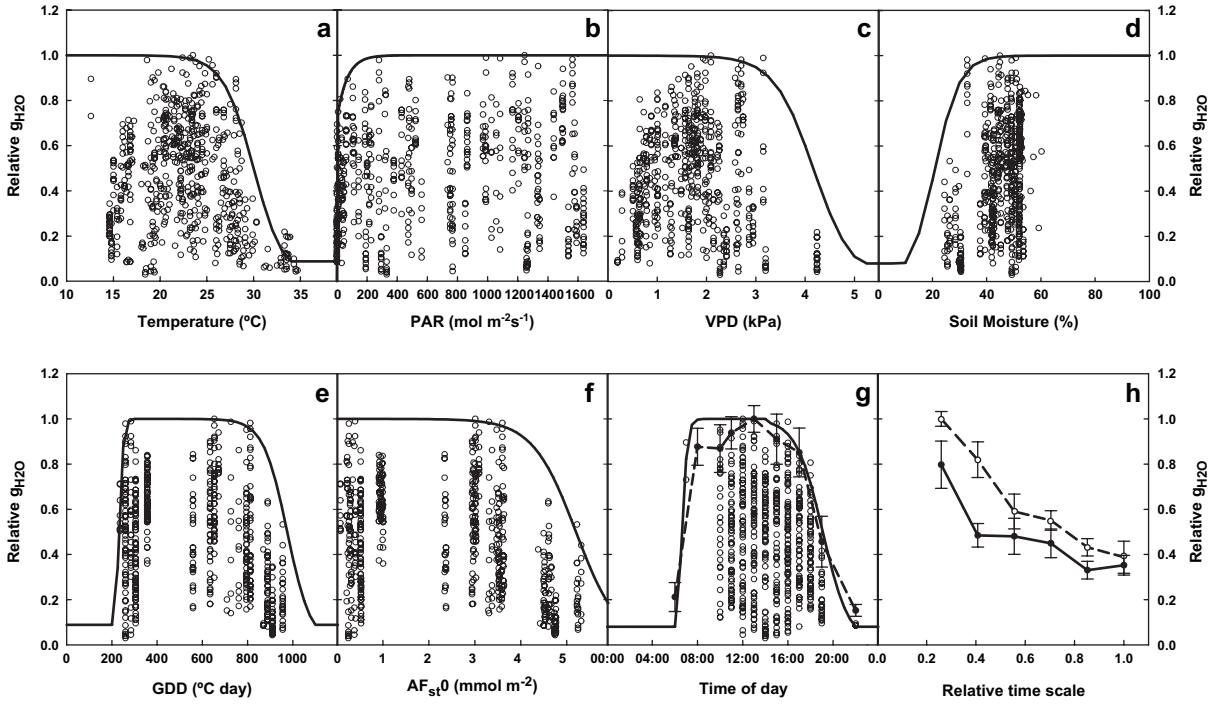

Fig. 1. Boundary line analysis underpinning multiplicative stomatal conductance model derived for field-grown lettuce (*Latuca sativa* L. cv. Paris Island). (a) Leaf temperature (°C); (b) PAR (μmol m^{-2} s^{-1}); (c) VPD (kPa); (d) SM (%); (e) GDD (°C days); (f) $AF_{st}0$ (mmol m^{-2}); (g) time of day; and (h) correction factor derivation to account for ozone effects.

3. Results

3.1. Impacts of ozone on yield

No significant differences in yield were found between replicate chambers within treatments, so data were analysed on the assumption that plants raised in the same chamber were as likely to be as different as those from replicate chambers. Although yield depended on cultivar ($P < 0.001$), both genotypes responded similarly to ozone (ozone × genotype, $P > 0.05$). Yield was significantly ($P < 0.001$) reduced by exposure to NFA + 50 nmol mol^{-1} O_3 (see Table 1).

3.2. Stomatal conductance model

Employing a calculated g_{max} value of 198 mmol H_2O m^{-2} s^{-1} and a corresponding g_{min} value of 0.09, the derived multiplicative model explained 51% of the variation in measured stomatal conductance data ($r^2 = 0.51$; $P < 0.001$) and 70% of the variation in hourly average stomatal conductance data ($r^2 = 0.70$; $P < 0.001$), with the model exhibiting a tendency to overestimate values of stomatal conductance in both cases (Fig. 2a,b).

Measurements of g_{H2O} exhibited considerable plant-to-plant variation as revealed by the standard errors for hourly

Table 1
Summary of environmental conditions and plant performance at the two field study sites

	Heraklion, Crete (2004)	Close House, U.K. (2003)			
Timing	17th March–6th May	1st July–27th August			
Environmental conditions					
PAR (μmol m^{-2} s^{-1})	54–1715	50–1358			
T (°C)	3–37.5	2.7–37.2			
VPD (kPa)	0.1–5.0	0.1–2.9			
Ozone exposure[a]		NFA	NFA + 25	NFA + 50	NFA + 75
Average daily maximum hourly [O_3] (nmol mol^{-1})	41.2	58.1	69.9	76.9	104.2
Average [O_3] (nmol mol^{-1})	30	38.4	43.8	46.7	57.1
AOT20 (nmol mol^{-1} h)	11.0	16.2	20.8	23.0	31.7
Stomatal conductance					
g_{max} (mmol H_2O m^{-2} s^{-1})	198	Not determined			
Average yield (shoot dwt (g))		NFA	NFA + 25	NFA + 50	NFA + 75
Paris Island	Not determined	19.8 ± 1	15.5 ± 0.6	16.0 ± 0.8	15.8 ± 0.7
Salad Bowl	Not determined	16.4 ± 3	13.0 ± 0.2	13.4 ± 0.4	13.0 ± 0.5

[a] Daytime (PAR ≥50 μmol m^{-2} s^{-1}).

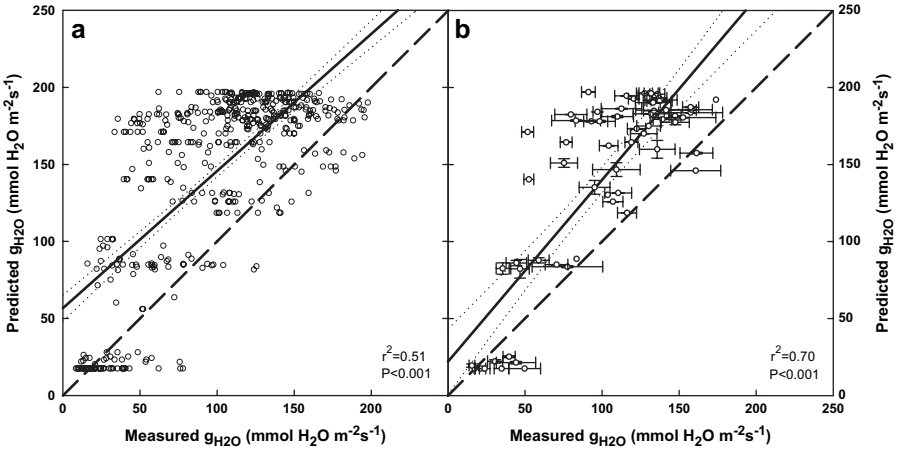

Fig. 2. Modelled versus measured stomatal conductance. Dashed line depicts 1:1 relationship.

average g_{H2O} (Fig. 2b). Diel measurements of stomatal conductance recorded in the field at the beginning of the experiment showed: (i) maximum g_{H2O} values were sustained during the morning; and (ii) g_{H2O} declined rapidly after c. 18:00 h. This behaviour was observed over the entire experimental period and was consistent with data recorded during controlled environment studies as represented in Fig. 1g by the dashed line.

Higher average values of g_{H2O} were recorded at the beginning of the experiment in Crete, decreasing from the 24th April (725.5 °C days) as the leaves aged. A summary of the functions used to derive the model is presented in Table 2.

3.3. Weather at the study sites

The stomatal conductance model was developed from measurements taken in the field during an experiment performed in spring in Crete (17th March– 6th May 2004) while exposure-response relationships were established during an open-top chamber experiment conducted in summer at a rural site in Northern England (1st July–27th August 2003). Table 1 provides a summary of the environmental conditions at the field sites during experimentation. Climatic conditions were broadly comparable despite the geographic discrepancies. The greatest differences were recorded in the range of VPD (see Fig. 3), although differences were restricted to a relatively short period (12% of the daylight hours), when the VPD experienced in Crete exceeded the maximum registered at Close House [2.9 kPa]).

Table 2
Equations derived from boundary line analysis relating to the impacts of environmental variables on relative stomatal conductance

$g_T = 1/(1 + 1.1 * 10^{-9}\ e^{0.61T})$;
$g_{PAR} = 1 - 0.28\ e^{-0.015\ PAR}$;
$g_{VPD} = 1/(1 + 3 * 10^{-5}\ e^{2.5\ VPD})$;
$g_{SM} = 1/(1 + 100\ e^{-0.22\ SM})$;
$g_{phen} = 1/(1 + 3.4 * 10^{10}\ e^{0.1\ GDD})$ if GDD <300;
$g_{phen} = 1/(1 + 9.17 * 10^{-9}\ e^{0.02\ GDD})$ if GDD >300;
$g_{AFst0} = 1/(1 + 1.6 * 10^{-5}\ e^{2.12\ AFst0})$;
$g_{time} = 1/(1 + 10^{12}\ e^{-100\ time})$ if time <14:00;
$g_{time} = 1/(1 + 9 * 10^{-8}\ e^{20.4\ time})$ if time >14:00.

3.4. Comparison of ozone exposure indices

The strongest correlation between yield and a variety of ozone indices was obtained using the accumulated flux (AF_{st}) employing thresholds of 1 nmol m^{-2} s^{-1} or 0 nmol m^{-2} s^{-1}, explaining 82% ($P < 0.05$) and 81% ($P < 0.01$) of the variation in yield, respectively (see Fig. 4) However, all regressions between yield and AF_{st} employing cut-off thresholds up to 4 nmol m^{-2} s^{-1} were statistically significant (see Table 3). Poorer relationships were revealed for concentration-based indices (AOTX) (see Fig. 4 and Table 3), with the strongest AOT index (AOT20) explaining only 58% of the variation in yield ($P < 0.05$). 'Modified' exposure-based indices (mAOT20) resulted in marginal improvement in relationships ($r^2 = 0.59$, $P < 0.05$).

4. Discussion

The present study on lettuce revealed data consistent with investigations on wheat (Danielsson et al., 2003; Kaminska et al., 2005) and potato (Pleijel et al., 2004), where improved relationships between yield losses and flux-based indices have been observed compared with concentration-based indices. The adoption of a low or zero cut-off value (i.e. flux threshold) – in the case of the present study, $AF_{st}1$ or $AF_{st}0$ – resulted in the strongest flux-response relationships. The dataset presented do not lend mathematical support for the inclusion of a flux threshold in the calculation of critical fluxes. However, the incorporation of a cut-off flux threshold significantly improves modeled predictions for wheat (Danielsson et al., 2003; Kaminska et al., 2005) and potato (Pleijel et al., 2004) and is consistent with biological understanding that plant foliage exhibits an intrinsic capacity to: (i) detoxify a fraction of the environmentally-relevant ozone flux; and (ii) repair a part of the oxidative damage resulting from the reaction of ozone taken-up into the leaf interior with key cellular constituents (see Barnes et al., 2002). Yet, the flux

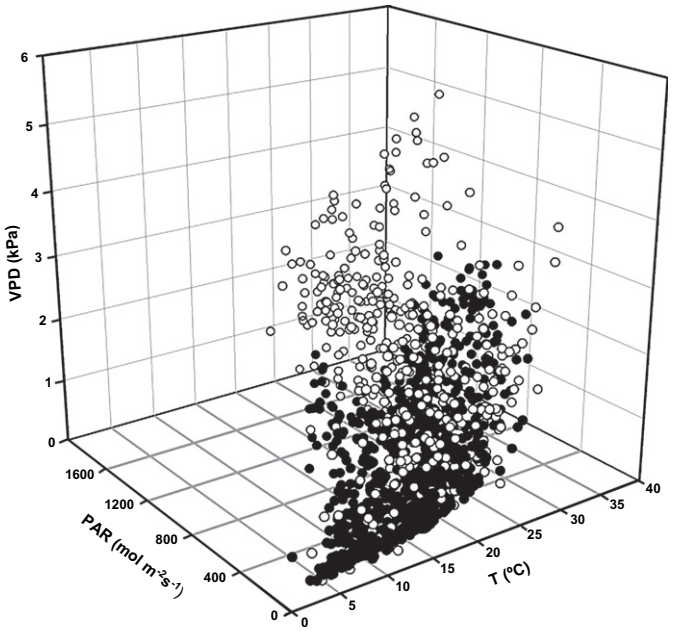

Fig. 3. Scatter plot illustrating measured temperature (T), vapour pressure deficit (VPD) and photosynthetically active radiation (PAR) over the course of ambient air experiments (open circles) and open-top chamber experiments (filled circles).

threshold for lettuce in this experiment appears to be considerably less than that derived from equivalent datasets for wheat and potato. Does this reflect a differential capability in lettuce to detoxify/repair ozone injury? Or is this simply a reflection of weaknesses in this particular dataset? Detailed investigations on cell wall localised-detoxification systems in *Lactuca sativa* cv. Paris Island suggest no evidence of reduced ozone scavenging capacity in comparison with other species (Goumenaki and Barnes, in prep.). Moreover, the stomatal conductance dataset employed for the derivation of the multiplicative algorithm used to predict ozone uptake was not as extensive it might have been, and missing data (e.g. at leaf temperatures below 14 °C; between sunrise and early morning; at extreme VPDs or at soil moisture contents below 25%) mean that an element of extrapolation was required to complete the necessary boundary line analysis. Given the importance of VPD, in particular, in governing stomatal conductance and the need for as robust definition as possible of the reaction of stomatal conductance to VPD to facilitate modelling of ozone uptake under Mediterranean conditions, there is a need to build-upon the existing dataset to derive an improved algorithm for the calculation of ozone uptake by lettuce (as well as other Southern European crops) under the range of conditions experienced over the Mediterranean growing season. The maximum stomatal conductance (g_{max}) derived from the dataset was relatively low (198 mmol H_2O m^{-2} s^{-1}) and although consistent with other g_{max} data published for lettuce (Kim et al., 2004; Bie et al., 2004; Calatayud et al., 2002) further data need to be collected to verify this key parameter prior to scaling-up exercises. One point worthy of highlighting is the difference in the diel function (g_{time}) derived from field-based measurements for lettuce compared with those currently adopted for wheat and potato (UNECE, 2004). Our data revealed stomatal conductance for lettuce to increase from a night-time g_{min} to a sustained optimum between 08:00 and 14:00, followed by a decline in the afternoon until sunset – a pattern of change in conductance in lettuce supported by independent measurements made during extensive controlled environment studies on multiple cultivars (Fig. 1g). The time factor, g_{time}, can be considered redundant for this particular dataset, as inclusion resulted in marginal differences in model predictions. Moreover, model combinations using time of day and/or SumVPD (as it appears in the UNECE Mapping Manual 2004, using a SumVPD critical threshold of 19.44 kPa) yielded variation in the robustness of model predictions (in terms of the resulting r^2) of <0.5%. Given the lack of experimental data at the beginning and at the end of each measurement day in the present dataset, it did not prove possible to check the incorporation of such factors in a robust manner. Several authors stress the need to incorporate a function reflecting the afternoon depression in stomatal conductance under Mediterranean conditions. This has most often been achieved by

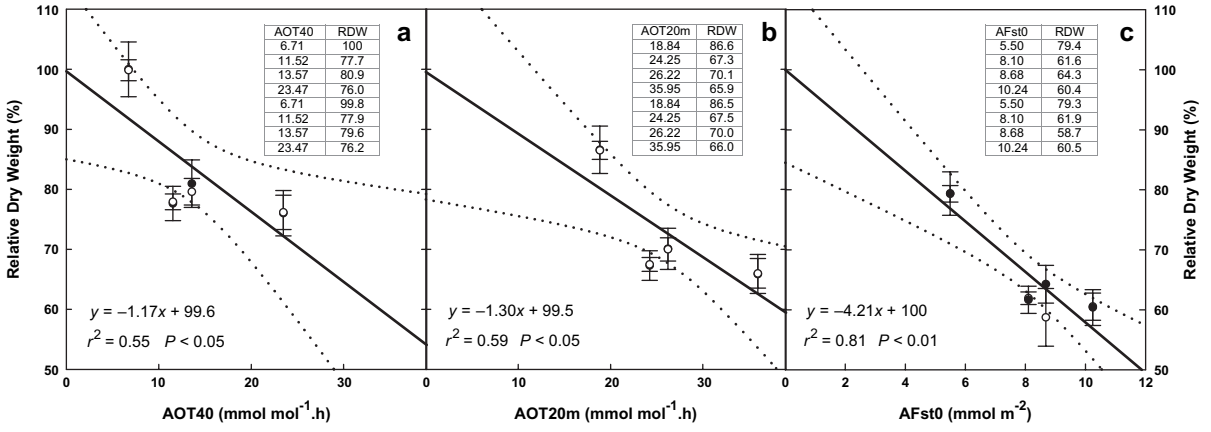

Fig. 4. Exposure-response and dose-response relationships (a) accumulated exposure to ozone concentrations over a threshold of 40 ppb (AOT40, ppm h) during daylight hours; (b) modified accumulated exposure to ozone over a threshold of 20 ppb (AOT20m, ppm h) during daylight hours; and (c) accumulated stomatal ozone flux during daylight hours employing no threshold ($AF_{st}0$, mmol m^{-2}). Tables show values, since some points used for regression derivation overlapped, Open circles represent data for 'Paris Island'; Filled circles represent data for 'Salad Bowl'. Bars represent the standard error of the mean.

Table 3
Regression analysis summary of relationships between ozone-induced shifts in plant dry weight (i.e. yield) and a variety of concentration- and flux-based ozone indices

	$AF_{st}0$	$AF_{st}1$	$AF_{st}2$	$AF_{st}3$
$AF_{st}Y$	$y = -4.2x + 100$ $r^2 = 0.81$ ($P < 0.01$)	$y = -4.9x + 99.8$ $r^2 = 0.82$ ($P < 0.05$)	$y = -5.9x + 99.8$ $r^2 = 0.73$ ($P < 0.01$)	$y = -6.9x + 99.8$ $r^2 = 0.62$ ($P < 0.05$)
AOTX	AOT20 $y = -1.0x + 99.5$ $r^2 = 0.58$ ($P < 0.05$)	AOT30 $y = -1.1x + 99.6$ $r^2 = 0.58$ ($P < 0.05$)	AOT40 $y = -1.2x + 99.6$ $r^2 = 0.55$ ($P < 0.05$)	AOT50 $y = -1.3x + 99.7$ $r^2 = 0.50$ ($P = 0.05$)
mAOTX	mAOT20 $y = -1.0x + 99.5$ $r^2 = 0.59$ ($P < 0.05$)	mAOT30 $y = -1.1x + 99.6$ $r^2 = 0.58$ ($P < 0.05$)	mAOT40 $y = -1.2x + 99.6$ $r^2 = 0.56$ ($P < 0.05$)	mAOT50 $y = -1.3x + 99.7$ $r^2 = 0.50$ ($P < 0.05$)

incorporation of a 'time of day' function or SumVPD (Grüters et al., 1995; Pleijel et al., 2002; Danielsson et al., 2003; UNECE Mapping Manual, 2004). Indeed, Jones (1983) points-out the necessity to include the time of day as an independent variable in multiple regression models predicting g_{H2O}.

This work should be regarded as a first attempt to develop dose-response relationships for a key horticultural crop in the Mediterranean area. Additional work will be required to build-upon and validate this dataset.

5. Conclusions

The study lends additional support to a move away from concentration-based indices to flux-based indices in a bid to improve pan-European ozone risk assessment procedures.

Acknowledgements

This work benefited from support and funding provided by TEI: Crete, The Royal Society, EU and the UK Department of the Environment, Food and Rural Affairs. IGF was supported by a Fellowship via JB's EU Framework 5 Marie Curie Training Site (HPMT-CT-2001-00219).

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3. ESTABLECIMIENTO DE RELACIONES DOSIS-RESPUESTA PARA EL TRIGO DE INVIERNO

González Fernández I, Kaminska A, Dodmani M, Goumenaki E, Quarrie S, Barnes J. (2009). Establishing ozone flux-response relationships for winter wheat yield. Atmospheric Environment, submitted.

Resumen

El estudio que se presenta en este artículo tuvo tres objetivos principales. El primero consistió en explorar los efectos de la contaminación por ozono (O_3) en la producción y la calidad de variedades comerciales de trigo de invierno. El segundo objetivo fue desarrollar una parametrización del modelo de conductancia estomática para el trigo de invierno y compararla con la parametrización existente para el trigo de primavera. El tercer objetivo fue comparar las relaciones de exposición- y dosis-respuesta desde el punto de vista del análisis de riesgo de daños provocados por el O_3, y explorar las implicaciones que la variabilidad intraespecífica pueda tener en el cálculo de los flujos estomáticos de O_3.

Quince variedades de trigo de invierno fueron expuestas a cuatro niveles de O_3 en cámaras de techo descubierto. Se utilizaron tres cámaras por cada tratamiento de ozono. Durante el experimento, se tomaron medidas de conductancia estomática durante el ciclo de vida de la hoja bandera en un rango variado de condiciones climáticas.

Se encontró una elevada variabilidad intra-específica en la respuesta de las plantas frente al O_3 en términos de producción. A pesar de esta varabilidad, los efectos estuvieron inversamente relacionados ($R^2 = 0.63$, $P < 0.001$) con la exposición al O_3 acumulada por encima de 40 ppb durante las horas diurnas (AOT40). Los efectos negativos del O_3 sobre la producción se debieron principalmente a una reducción en el peso de granos producidos por planta.

Las funciones que describen la influencia de las variables ambientales sobre la conductancia estomática del trigode invierno resultaron ser ligeramente distintas a las que se utilizan en la actualidad para calcular los impactos del O_3 sobre la producción de cereal en Europa basadas en el trigo de primavera. Se encontró que existía una elevada variabilidad intra-específica en la influencia del estado fenológico en la conductancia

estomática. Por esta razón, se optó por determinar una función de fenología que reflejase el comportamiento general de las variedades de trigo lo que se tradujo en una reducción de la capacidad de predicción del modelo de conductancia (R^2 = 0.49, P< 0.001, considerando las 15 variedades).

Dada la elevada variabilidad encontrada en el comportamiento estomático, el cálculo de los flujos de O_3 absorbido no supuso una mejora frente al AOT40 a la hora de predecir los impactos del O_3 en el trigo de invierno. El estudio destaca la necesidad de utilizar herramientas para el cálculo de los riesgos causados por el O_3 específicas para cada tipo de vegetación con el fin de poder modelizar y cartografiar los impactos del O_3 a escala regional.

ESTABLISHING OZONE FLUX-RESPONSE RELATIONSHIPS FOR WINTER WHEAT YIELD

Running title: O_3 dose and effects on winter wheat genotypes

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Key words: risk assessment, modelling ozone uptake, winter wheat, grain yield and quality, dose-response relationships.

Abstract

The work outlined in this paper had three principal objectives. The first was to explore the effects of ozone pollution on grain yield and quality of commercially-grown winter wheat cultivars. The second was to derive a stomatal ozone flux model for winter wheat and compare with those already developed for spring wheat, and the third, was to compare exposure- and flux - response approaches from a risk assessment perspective, and explore the implications of genetic variation in modelled ozone flux.

Fifteen winter wheat cultivars were grown in triplicate open-top chambers where they were exposed to four controlled levels of ozone. During fumigation, stomatal conductance measurements were made over the lifespan of the flag leaf across a range of environmental conditions. Although significant intraspecific variation in 'ozone sensitivity' (in terms of effects on yield) was identified, accumulated hourly averaged ozone exposure above 40 ppb during daylight hours (AOT40) was inversely related to yield ($R^2 = 0.63$, $P < 0.001$) across the dataset. The adverse effect of ozone on yield was principally due to a decline in seed weight. Algorithms defining the influence of environmental variables on stomatal uptake were subtly different from those currently in use, based on data for spring wheat, to map ozone impacts on pan-European cereal yield. Considerable intraspecific variation in phenological effects was identified. This meant that an 'average behaviour' had to be derived and this reduced the predictive capability of the derived stomatal flux model ($R^2 = 0.49$, $P < 0.001$, 15 cultivars included). Indeed, given the intraspecific variability encountered, the flux model that was derived from the full dataset was no better in predicting O_3 impacts on wheat yield than was the exposure-based AOT40 index. The study highlights the need to use ozone risk assessment tools appropriate to specific vegetation types when modelling and mapping ozone impacts at the regional level.

1. Introduction

Although the frequency, intensity and duration of ozone pollution episodes have been reduced in the Northern hemisphere over the past decade via the introduction of increasingly stringent emission control measures, 'background' levels of this air pollutant are expected to continue to rise at a rate of 0.5-2.0% per annum for the foreseeable future – largely as a result of rapid growth and industrial development in parts of the Southern hemisphere (Vingarzan, 2004). This gives cause for concern (Royal Society, 2008), as the gas is phytotoxic and current levels of the pollutant are known to be high enough to cause visible foliar injury, reduced growth and yield, accelerated senescence of foliage and altered sensitivity to a range of biotic and abiotic stresses in many common agricultural and horticultural crops (Turcsànyi *et al.*, 2000; Ashmore, 2005; Feng & Kobayashi, 2009). Present-day models predict that ground-

level ozone concentrations could rise 20-25% between 2015 and 2050, and further increase by 40-60% by 2100, if current emission trends continue (Meehl *et al*., 2007). Losses in agricultural production attributable to ozone pollution are valued[3] at *c.* € 7.5 billion across Europe and North America (Ashmore, 2005). Given the scale of the losses there would appear to be a clear need for the development of risk assessment tools to facilitate the modelling of impacts on specific crops and assist strategic policy development to curb emissions. In Europe, a 'Critical Levels' approach has been adopted for ozone risk assessment (see Fuhrer & Booker, 2003; UNECE, 2004). Initially, an exposure-based approach was employed based on the linear decline in crop yield resulting from cumulative exposure to ozone above a threshold of 40 ppb (AOT40) during the three months of the growing season that the crop is most active in the field (Fuhrer *et al.*, 1997). However, this approach was recognised to suffer several serious limitations, and the introduction of models delivering an estimate of ozone uptake over the lifespan of the crop (Emberson *et al.*, 2000; Grunhage *et al.*, 2000) have facilitated an improved approach where effects can be assessed in relation to ozone uptake (i.e. effective biological dose) rather than exposure (UNECE, 2004). Robust ozone flux-response relationships are now available for mapping and modelling the risks posed by past, present and future ozone concentrations to spring wheat and potato (Pleijel *et al*., 2007), and work on other crops is underway e.g. grapevine (Soja *et al.*, 2004), lettuce (Goumenaki *et al*., 2007), clover (Mills *et al*., 2003; Gonzalez-Fernandez *et al*., 2008), sunflower and tomato (Emberson *et al.*, 2005). Whilst there is a general consensus that the flux modelling approach constitutes a significant improvement on previous pan-European ozone risk assessment approaches, there is recognition that current models could be improved even further by the inclusion of (1) an ozone detoxification algorithm rather than the employment of an empirically-derived flux-threshold (Musselman *et al*., 2006) (2) consideration of effects on crop quality as well as yield (see Gonzalez-Fernandez *et al*., 2008) and (3) potential regionalisation of modelling approaches to account for local edaphic and plant-specific variances (Fuhrer & Booker, 2003; Fiscus *et al.*, 2005).

Because of the importance of wheat production to the European economy much

[3] Based on 2007 prices (Europa Rapid Press Releases Reference: IP/07/1977, 20/12/2007)

attention has focused on assessing the risks posed to this crop by ozone pollution. Across the EU-27, wheat occupies the greatest area (around 25 million ha are grown each year), exhibits the largest production (*c.*130 million tonnes of grain in 2007) and delivers the greatest economic value (worth *c.* €33.2 billion based on end-2007 escalated prices due to rising fuel costs and changing production strategies). It is thus surprising, given the overwhelming commercial importance of winter wheat across the EU-27, as well as in other parts of the world, that the vast majority of ozone–related research on wheat has focused on spring-sown genotypes (see meta-analysis by Feng *et al*., 2008). This extensive body of work reveals modern hexaploid wheat (*Triticum aestivum* L.) to be one of the most ozone-sensitive crops identified to date (Mills *et al*., 2007); exposure to a 7-h seasonal mean [O_3] of 72 ppb reducing wheat grain yield by *c.* 30%. However, wheat genotypes are known to exist considerable, heritable, variation in sensitivity (i.e. ozone impacts on grain yield) between cultivars (Barnes *et al*., 1990; Velissariou *et al*., 1992; Barnes *et al*., 1999; Biswas *et al*., 2008) although the genetic basis for this variation remains to be dissected and understood (see Quarrie *et al*., 2006).

The purpose of the present study was to (1) explore the extent of genetic variation in the effects of O_3 on grain yield and quality-related characteristics in commercially-grown winter wheat, targeting parents of partially-mapped double haploid populations with a view to using the information to probe quantitative trait loci conferring ozone resistance; (2) derive a stomatal flux model (*sensu* Emberson *et al*., 2000) for winter wheat to enable comparison with similar models developed for spring wheat and compare exposure- *versus* flux - response approaches; and (3) explore the extent of genetic variation in modelled ozone flux and highlight the implications for pan-European modelling approaches.

2. Materials and methods

2.1 Plant cultivation

Ten-day-old seedlings of eleven commercially-grown UK cultivars of winter wheat (Avalon, Claire, Consort, Deben, Hereward, Hobbit, Riband, Rialto, Soissons, Spark and Steadfast), and four commercially-grown Polish cultivars of winter wheat (Kamila, Kobra, Korweta and Rywalka), were transferred in November 2003 to pots containing

15 dm^3 of potting compost (Levington John Innes No. 2, compost: peat: sand [2:1:1]; incorporating John Innes Base fertilizer [5:7:10 NPK] plus lime). Plants were transferred to unprotected cold frames adjacent to a glasshouse and thinned to 16 seedlings per pot. On 1 May 2004, three pots per cultivar were randomly assigned to each of twelve open-top chambers (three OTC replicates per ozone treatment) situated at Newcastle University's Close House Field Station at Heddon-on-the-Wall, Northumberland, in Northern England (National Grid Reference NZ 128659, latitude 54º59'N, longitude 1º48'W, elevation 30 m.a.s.l.). The 8.5 m^2 of floor space in each OTC was divided into three, and one pot of each cultivar randomised within each division. All OTCs were ventilated with non-filtered air (NFA). On 7 May, ozone was injected in to the ductwork ventilating nine of the twelve chambers (with ozone treatments randomly assigned to chambers) to achieve four targeted daytime (7 h d^{-1} (10.00-17.00) treatments: NFA, NFA+25 ppb O_3, NFA+50 ppb O_3 and NFA+75 ppb O_3) delivering a seasonal (6 May to 5 August) accumulated hourly mean ozone exposure above 40 ppb (AOT40) of 2158, 9327, 13564 and 18919 ppb.h, respectively. Each OTC was ventilated (day and night) *via* a perforated annulus positioned 1.3 m above ground-level via a fan supplying sufficient particulate-filtered air (non-filtered air: NFA) to achieve two air changes min^{-1} in each chamber. Ozone was generated from oxygen by electric discharge (Model SGC22, Pacific Ozone Technology, Benicia, California 94510 USA) and supplied to O_3 chambers following dilution in a stream of clean compressed air. Ozone levels were logged and controlled using a feedback regulation system based around a motorized voltage regulator, rather than mass flow controllers. Further details of the OTC facility and ozone control systems are provided in Gonzalez-Fernandez *et al*.(2008).

Plants were watered as required throughout the experiment to ensure a soil moisture content sufficient to support uninterrupted growth, and were sprayed with a combination of pesticides ('Radar' and 'Malathion 60'). Plants were fertilised at fortnightly intervals using liquid fertilizer 'Nitro-Top': 33.5% N.

2.2 Measurement campaign

2.2.1 Environmental variables

Ozone concentrations were monitored at canopy height in the centre of each OTC via a solenoid-based time-share system using a photometric analyser (Dasibi Environmental

Corporation (Dasibi) Model 1008 Ultraviolet (UV) Photometric Ozone Analyzer, Series 1008-PC; Dasibi Environmental Corporation, 515 W. Colorado St., Glendale , California 91204-1101), serviced weekly and cross-checked against a similar unit calibrated against NPL standards.

Photosynthetically-active radiation (PAR), air temperature and relative humidity were recorded with cross-referenced sensors using a combination of loggers (Sentry Intelisys Ltd, Manchester, UK. and Delta-T Devices Ltd, Cambridge, UK.), and all environmental measurements were made at the position occupied by the flag leaf (in one OTC).

2.2.2 Stomatal conductance

Adaxial stomatal conductance to water vapour (g_{H20}) was measured in the mid-region of the flag leaf at regular intervals over it's lifespan across a range of monitored environmental conditions. Measurements were made with cross-referenced Delta-T Devices AP4 Porometers (Delta-T Devices, Ltd, Cambridge, UK) and to express data on a projected leaf area basis all measurements were multiplied by 1.43 to account for the total:adaxial surface conductance ratio exhibited by the flag leaf of wheat (Amundson *et al*., 1987; Araus *et al*., 1989).

Web-based databases were used to identify all peer-reviewed literature reporting stomatal conductance measurements for wheat raised in the field. Following the quality criteria outlined in Pleijel *et al.* (2007) 17 field studies (spanning Europe, America and Asia, and covering 27 cultivars of spring and winter wheat) were identified from which maximum stomatal conductance was calculated. Of those identified, 3 of the studies on spring wheat (delivering data for 5 cultivars) contributed to the wheat flux model derivation in the UNECE Mapping Manual (UNECE, 2004). Stomatal conductances to CO_2 were multiplied by 1.56 to account for the difference in diffusivity between CO_2 and H_2O in air (Larcher, 2003), then all stomatal conductance values were normalised on a projected leaf area basis.

2.3 Stomatal conductance model and estimation of ozone flux ($AF_{st}Y$)

Measurements of g_{H20} and meteorological parameters were used to parameterise a Jarvis-style (Jarvis, 1976) model where g_{H2O} is estimated from a multiplicative function

describing the manner in which g_{H2O} responds to key species-specific and environmental variables via effects on maximum stomatal conductance (g_{max}). Maximum stomatal conductance was calculated based on the average g_{H20} above the 90th percentile (*sensu* Gonzalez-Fernandez *et al.*, 2008). f_{min} was calculated as the average of g_{H2O} values below the 10th percentile for night-time measurements. The relationship between g_{H2O} and the climatic variables was analysed by applying a boundary line approach to a data cloud containing 1,983 measurements of stomatal conductance made on flag leaves borne on the primary shoot over a range of conditions over the leaf life-span (*sensu* Emberson *et al*., 2000). This technique assumes the line connecting data points at the outer margin of the data cloud depicts the maximum possible g_{H2O} for a given value and thus the functional dependency between the plotted variables (Jarvis, 1976; Emberson *et al.*, 2000). A minimum stomatal conductance (f_{min}) was considered in the model based on 452 g_{H2O} measurements taken over-night during the course of the experiment.

Factors considered to modify g_{max} were: photosynthetically-active radiation (light), air temperature (temp), vapour pressure deficit (VPD), phenology (phen) and accumulated stomatal ozone flux ($AF_{st}0$) (Pleijel *et al*., 2007). Soil water potential (SWP) was not considered, as plants were kept well-watered throughout. The influence of VPD on stomatal conductance in the afternoon was also considered using the ? VPD function (*sensu* Pleijel *et al.*, 2007). A phenological weighting factor, used to describe the reduction in g_{H2O} as leaves age and senesce, was determined using thermal time (tt) and for this purpose, the mean value between maximum and minimum daily temperature was used, employing a basal temperature of 0^{o}C (*sensu* McMaster & Wilhelm, 1997). Circadian influences on stomatal conductance resulting in partial stomatal closure during the afternoon were also taken into account by deriving f_{time} from boundary line analysis of diel g_{H2O} (*sensu* Danielsson *et al.*, 2003). The f_{phen} function includes a plateau of maximum activity around anthesis before decreasing towards the end of the experiment. The f_{AFst} function takes into account the O_3 cumulated flux effect, operating from three weeks before anthesis up to the end of the experiment. The f_{light}, f_{temp} and f_{VPD} functions account for short-term effects of photosynthetically-active radiation (light), air temperature (T) and vapour pressure deficit (VPD) on g_{max}. The time window selected for the flag leaf accumulation of O_3 flux was set from 10 May to 14 July 2004, from tt = -379ºC day (about three weeks before the average date of anthesis) to 518ºC

day after anthesis (about four weeks after average date of anthesis), by which time most of the genotypes were showing flag leaf senescence.

Calculations of O_3 uptake per unit leaf projected area (F_{st}) were based on the guidelines provided in the CLRTAP Mapping Manual (UNECE, 2004)

$$F_{st}Y(nmol\,m^{-2}\ s^{-1}) = C_{O_3}(nmol\ m^{-3}) \cdot g_{H_2O}(m\,s^{-1}) \cdot D_{H_2O/O_3} \cdot \frac{r_c}{r_b + r_c} \qquad (1)$$

where, $r_c = \frac{1}{g_{H_2O} + g_{ext}}$; r_b = 11.76 s m^{-1}; g_{ext} = 0.0004 m s^{-1}; c_{O3} is the hourly O_3 concentration; r_c is the leaf surface resistance; r_b is the quasi-laminar resistance and g_{ext} is the leaf cuticular conductance. Ozone flux estimates were based on the hourly mean O_3 concentration and considered quasi-laminar and cuticular resistances. Values were calculated based on the difference in diffusivity between water vapour and O_3 in air; $D_{H2O/O3}$ = 0.613 (Nobel, 1999). The wind speed used for the quasi-laminar resistance was 5.5 m s^{-1}; representing the air flow in the OTCs employed for the investigation.

Stomatal flux was accumulated on an hourly basis over the life-span of the flag leaf

$$AF_{st}Y(mmol\,m^{-2}) = \sum \left| (F_{st} - Y(nmol\,m^{-2}\ s^{-1})) * 3600(s\,h^{-1}) * 10^{-6} \right| \qquad (2)$$

and the effect of different flux thresholds (*Y*) on dose-response relationships examined The predictive capacity of the derived stomatal flux model was compared with the existing DO_3SE model derived from data for Northern European spring wheat (UNECE, 2004).

2.4 Yield and yield components

Plants were harvested over a ten-day-period at full maturity (7-17 August 2004). The ears from the tallest plant in each pot were bagged separately from those of the remaining plants, and these were employed for subsequent detailed yield component analysis. Remaining ears were mechanically threshed, then grain number and weight recorded.

Measurements of grain protein content (GPC) and a-amylase (AA) activity were restricted to one ‘sensitive’ and one ‘resistant’ cultivar: ‘Rialto’ and ‘Hereward’, respectively. Grain was ground using a pestle and mortar, and the resulting flour sieved

(0.5 mm mesh size) prior to analysis. Total nitrogen content was determined by combustion using a LECO Model FP-428 C:N analyzer, calibrated with EDTA and data reported on the basis of 14% moisture content. Protein content was calculated by multiplying grain nitrogen content by 5.7 (Williams *et al.*, 1998). a-amylase activity was determined using a kit (Megazyme, Bray, Ireland) based on an assay described by McCleary and colleagues (2002). The Falling Number (FN), sometimes known as the Hagberg Index and a descriptor of the bread-making quality of wheat flour, is inversely proportional to AA activity (Olered, 1967).

2.5 Statistical analyses

Effects were tested by ANOVA, using SPSS v.12.0 (SPSS Inc., Chicago, USA), with a confidence level of 95% (P= 0.05). Normality and variance homogeneity were examined prior to every analysis. When necessary, Kruskal-Wallis or U Mann-Witney tests were used, otherwise statistical significance was determined using least significant difference test (LSD). First, positional and chamber effects were tested by ANOVA. No significant influences were detected, so data were analysed on the assumption that plants in replicate chambers were as likely to be as similar to, or as different from, plants within an individual chamber. Linear regressions were performed to examine exposure- or dose-response relationships (based on relative effects) using the principles described in Fuhrer (1994) where linear regressions are performed for each individual genotype and then each response value is divided by the intercept of its regression equation so that zero O_3 exposure or dose is always associated with no effect (100%). Exposure- and dose-response relationships considered all genotypes together, as well as 'sensitive' and 'resistant' cultivar groups separately. Graphs were drawn using SigmaPlot 9.0 (Systat Software, Inc).

3. Results

3.1 Stomatal conductance model

Equation 3 shows the g_{H2O} model delivering the best fit to the winter wheat dataset

$$g_{H_2O} = g_{\max} * \min\left\{f_{phen}; f_{O_3}\right\} * \min\left\{f_{light}; f_{time}\right\} * \max\left\{f_{\min}; (f_{temp} * f_{VPD} * f_{SWP})\right\} \quad (3).$$

Here, g_{H2O} represents the stomatal conductance to water vapour of the flag leaves expressed per unit projected leaf area (mmol m^{-2} s^{-1}); g_{max} represents maximum stomatal conductance; f_{phen} represents the influence on phenology on g_{max}; f_{O3} represents the effects of accumulated ozone stomatal flux ($AF_{st}0$ in mmol m^{-2}) on g_{max}, simulating flag leaf senescence; f_{light}, represents the influence of photosynthetically active radiation on g_{max}; f_{time} represents the influence of circadian rhythms on g_{max}; f_{min} represents minimum stomatal conductance; f_{temp} represents the influence of air temperature (^oC) on g_{max}; f_{VPD} represents the influence of vapour pressure deficit (kPa) on g_{max}; f_{SWP} represents the influence of soil water potential on g_{max}. For this well-watered experiment f_{SWP} was not considered (i.e. the value was set at 1).

Table 1 shows boundary line functions derived for winter wheat and provides a direct comparison with those derived, and currently in use for pan-European risk assessment, for spring wheat. Our winter wheat model explained 49% ($P< 0.001$) of the variation in measured stomatal conductance and up to 55% ($P< 0.001$) of the hourly averaged g_{H2O} (employed for estimating ozone flux). Figure 1 shows that the derived model tended to over-estimate stomatal conductance, and analysis of hourly average g_{H20} revealed the relatively low predictive capacity of the model to be due, at least in part, to plant-to-plant variability across the dataset.

Maximum stomatal conductance derived from the data cloud for winter wheat was 508 ± 21 mmol O_3 m^{-2} s^{-1} with considerable variation in g_{max} between cultivars; values ranging between 396 and 649 mmol O_3 m^{-2} s^{-1} across the 15 cultivars used in the present study (Table 2). Furthermore, the g_{max} recorded in the present study was not significantly different from the value (450 ± 52 mmol O_3 m^{-2} s^{-1}) derived previously for spring wheat from field studies (see Table 1). A meta-analysis (Table 3) of the peer-reviewed literature lends support to our observations, revealing considerable variation in g_{max} between cultivars, but no significant difference in g_{max} between spring and winter genotypes although winter wheat appears to consistently exhibit lower g_{max}, bar a single study. g_{max} measured in the present pot-based study (508 mmol O_3 m^{-2} s^{-1}) was higher ($P< 0.05$), rather than lower, than equivalent measurements reported from previous field studies (343 mmol O_3 m^{-2} s^{-1}).

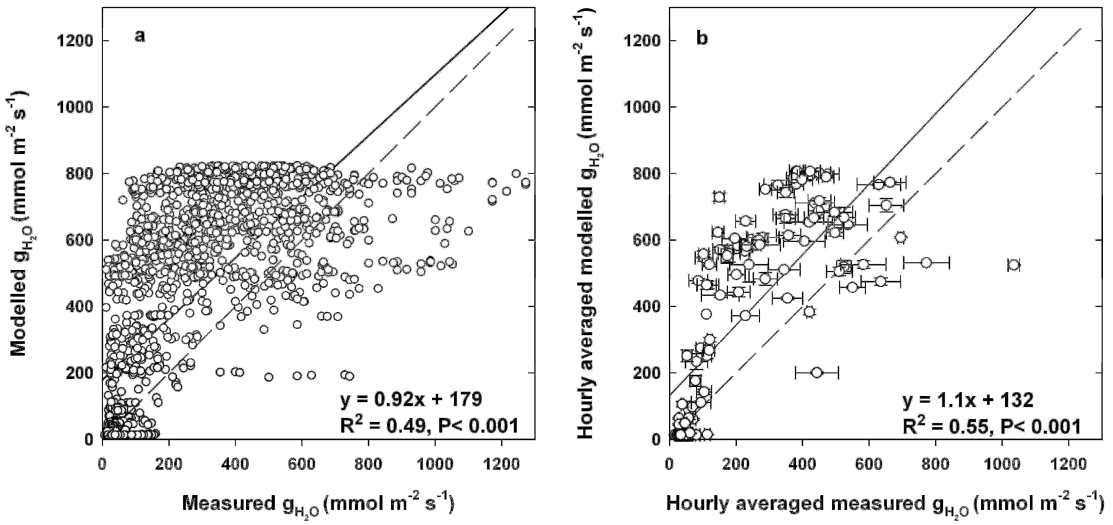

Figure 1. Linear regressions of modelled *versus* measured stomatal conductance (g_{H2O}) values: a) instantaneous g_{H2O}; b) Hourly averaged g_{H2O}, bars depicting standard errors. Solid lines represent the linear regressions. Equations, R^2 and P-values are also provided. Dashed lines represent the 1:1 reference line.

Parameter	Winter wheat (this study)	Spring wheat (UNECE, 2004)
Time window	Start: -379ºC day (three weeks before anthesis) End: 518ºC day (four weeks days after anthesis)	Start: -270ºC day (two weeks before anthesis) End: 700ºC day (40 days after anthesis)
g_{max}	508 mmol O_3 m^{-2} s^{-1}	450 mmol O_3 m^{-2} s^{-1}
f_{min}	0.016	0.01
f_{phen}	If tt ≤200 ºC day; y=1/(1+3.76*10^{-3} *exp(-0.021*tt)) If tt >200 ºC day; y=1/(1+2.51*10^{-4}*exp(0.0186*tt))	If tt = 270ºC day; y = 1 If 270 < tt ≤ 700ºC day; y=1-(0.8/700)*tt
f_{O3}	y = 1/(1+ exp(0.594*(AF_{st}0-15.24)))	Y = 1/(1+(AF_{st}0/11.5)^{10})
f_{light}	y = 1-exp(-0.015*PAR)	Y = 1 – exp(-0.0105*PAR)
f_{time}	If time<11:30; y = 1/(1+19795.3*exp(-30*time)) If time≥11:30; y=1/(1+8.28*10^{-9}*exp(25.25*time))	Not aplicable
f_{temp}	If 9.7ºC≤T≤39.1ºC; y = [((T-9.7)/15.3)*((39.1-T)/14.1)]$^{0.9216}$	If 12ºC≤T≤40ºC; y = [((T-12)/14)*((40-T)/12)]^{1}

	y = 0.016	y = 0.01
f_{VPD}	If VPD < 1.4 kPa; y = 1 If 1.4kPa ≤VPD≤ 3 kPa; y=((2.93-0.98*VPD)/1.6)+0.016 If VPD > 3 kPa; y = 0.016	If VPD < 1.2 kPa; y = 1 If 1.2 kPa ≤VPD≤ 3.2 kPa; y = ((3.17-0.99*VPD)/2) + 0.01 If VPD > 3.2 kPa; y = 0.01

Table 1. Parameterization of stomatal conductance model. Algorithms derived from boundary line analysis of the influence of key environmental and plant-specific factors on stomatal conductance. Time window for integration and boundary line equations in the winter wheat model based on observations made during OTC experiments; g_{max} represents maximum stomatal conductance; f_{min} represents minimum stomatal conductance; f_{phen} represents the phenological weighting factor; tt represents thermal time (ºC day); f_{O3} represents the effects of accumulated ozone stomatal flux ($AF_{st}0$ in mmol m^{-2}) on flag leaf senescence; light, photosynthetically active radiation; time, time of day (calculated as hour/24); temp, temperature (ºC); VPD, vapour pressure deficit (kPa).

Genotype	**g_{max}**	**SE**	**n**
Avalon	446	18	7
Claire	434	18	7
Consort	601	14	6
Deben	441	29	9
Hereward	546	41	8
Hobbit	519	30	8
Kamila*	587	46	6
Kobra*	546	61	5
Korwetta*	419	25	7
Rialto	469	28	8
Riband	649	31	6
Rywalka*	396	26	6
Soissons	628	31	6
Spark	512	65	6
Steadfast	461	55	7
Mean value	**508**	**21**	**102**
Median	**509**		

Table 2. Maximum stomatal conductance (g_{max}, mmol O_3 m^{-2} s^{-1}) derived from measurements made on 11 UK and 4 Polish cultivars of winter wheat. *Polish genotypes.

		Reference	g_{max} (mmol O_3 m^{-2} s^{-1})	Country	Genotype
WINTER WHEAT	EU	Müller *et al*., 2005	429	Germany	Orestis
		Tuba *et a*l., 1994	161	Hungary	MV16*
	WORLD	Bunce, 2000	434	USA	Coker*
		Jiang *et al*., 2000	159	China	Jing 411
		Jiang *et al*., 2000	362	China	Jingdong 8
		Jiang *et al*., 2000	331	China	JingnongS4055
		Jiang *et al*., 2000	208	China	Shi 4185
		Jiang *et al*., 2000	153	China	Nongda 3338
		Shen *et al*., 2002	803	China	n.a.
		Xue *et al*., 2002	429	USA	Arapahoe
		Xue *et al*., 2002	383	USA	Cheyenne
		Xue *et al*., 2002	374	USA	Karl 92
		Xue *et al*., 2002	337	USA	Scout 66
		Yu *et al*., 2004	239	China	n.a.
		Mean value	**343**		
		Median	**349**		
		Table 3 (cont.)			
SPRING WHEAT	EU	Ali *et al.*, 1999	613	Denmark	Cadeusa
		Araus *et al.*, 1989	444	Spain	Kolibri
		Araus *et al.*, 1989	345	Spain	Astral
		Araus *et al.*, 1989	336	Spain	Boulmiche
		Araus & Tapia, 1985	477	Spain	Kolibri
		Danielsson *et al.*, 2003	508	Sweden	Dragon*
		Del Pozo *et al.*, 2006	251	Spain	Alcalá
		Mulholland *et al.*, 1997	454	UK	Minaret*
		Pleijel *et al.*, 2005[1]	435	Sweden	Vinjett
	WORLD	García *et al.*, 1998	417	USA	Yecora Rojo
		Sato *et al.*, 2006	416	Syria	5 cultivars
		Wall *et al.*, 2000	472	USA	Yecora Rojo
		Mean value	**431**		
		Median	**440**		

Table 3. Meta-analysis of maximum stomatal conductance values (g_{max}) to ozone (normalised on a projected leaf area basis) reported in the literature for the flag leaf of modern hexaploid wheat (*Triticum aestivum* L.) and abstracted from field studies using the criteria outlined by Pleijel *et al.* (2007). Measurements made under optimum conditions of photosynthetically active radiation, vapour pressure deficit, temperature and soil humidity. n.a. information not published. [1]conference proceedings (not peer-reviewed). * Experiments employing OTCs.

3.2 Impacts of ozone on yield and yield components

Exposure to ozone significantly ($P < 0.001$) reduced the weight of grain recovered per plant. The loss in yield was predominantly due to a reduction in thousand grain weight (TGW) and the number of seed produced per plant (principally a consequence of a decline in grain number per ear). There was no significant change in the number of ears produced per plant. Not unexpectedly, there was significant (O_3*cultivar $P < 0.05$) variation between cultivars in the extent of the yield depression induced by ozone, and a group of six 'sensitive' cultivars was statistically discernible ($P = 0.01$) from a group containing six 'resistant' cultivars. The 'sensitive' group comprised Claire, Consort, Deben, Korweta, Rialto and Steadfast, which exhibited an average depression in grain yield of 32% in the NFA+75 treatment. In contrast, the 'resistant' group, comprising Hereward, Kamila, Kobra, Riband, Soissons and Spark, exhibited a 17 % depression in grain yield in the NFA+75 treatment. Strong linear ozone exposure [AOT40]-response relationships were evident for key yield determinants (Fig. 2). Several $AF_{st}Y$ indices were tested (Table 4). The strongest ozone flux – response relationships (for relative yield) employed a threshold of 14 nmol O_3 m^{-2} s^{-1} ($R^2 = 0.61$, $P < 0.001$), but there was little difference in the goodness of fit between thresholds ranging from 8 to 14 nmol O_3 m^{-2} s^{-1} ($R^2 = 0.54$ to 0.61). Ozone flux – response relationships (for relative yield) were, however, significantly ($P < 0.01$) improved through the use of a cut-off threshold above 4 nmol O_3 m^{-2} s^{-1}. Employing $AF_{st}14$ to the 'sensitive' genotypes improved the R^2 for the ozone flux – response relationship (for relative yield) to 0.78 ($P < 0.001$). The loss in yield induced by ozone appeared to be partially offset by an increase in grain crude protein content (Fig. 3). However, protein yield per plant was not significantly affected by ozone. a-amylase activity was also positively related with O_3 exposure (Fig. 4) but 'sensitive' and 'resistant' genotypes appeared to respond similarly.

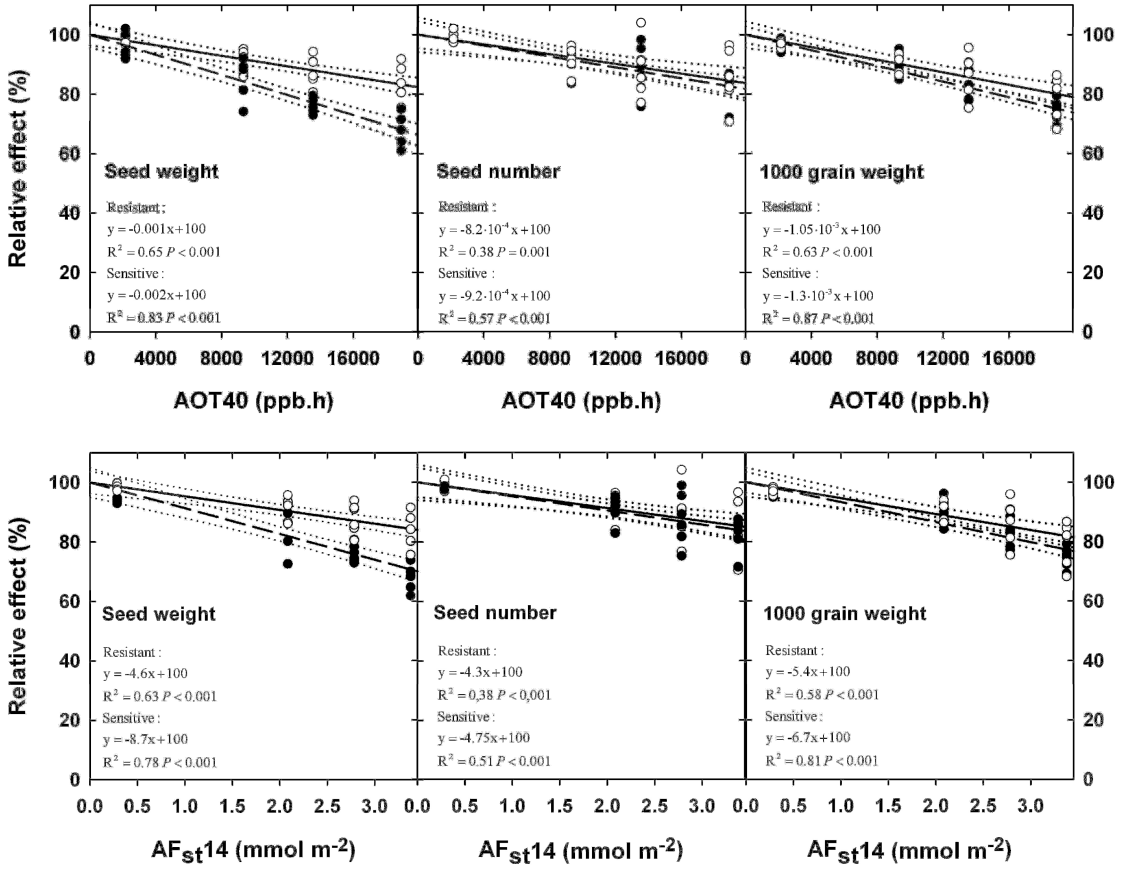

Figure 2. Ozone exposure and dose effects on yield components (seed weight per ear, seed number per ear and thousand grain weight) of winter wheat fumigated with ozone in open-top chambers. Ozone exposure was expressed as AOT40 (ppb.h), accumulated ozone exposure over a threshold of 40 ppb. Ozone dose was expressed as accumulated stomatal flux of ozone above a threshold of 14 nmol m^{-2} s^{-1} ($AF_{st}14$ (mmol m^{-2})). Yield components are expressed in a relative scale. Filled circles represent sensitive genotypes (Claire, Consort, Deben, Korwetta, Rialto, Steadfast), open cicles resistant genotypes (Hereward, Kamila, Kobra, Riband, Soissons, Spark). Dashed line is the linear regression with 95% confidence intervals for the sensitive group and solid line is the linear regression for the resistant group. Equations, R^2 and P-values of the regressions are also presented.

	AOT40	$AF_{st}0$	$AF_{st}4$	$AF_{st}6$	$AF_{st}8$	$AF_{st}10$	$AF_{st}12$	$AF_{st}14$	$AF_{st}16$
All	y = -0.001x +100 R^2 = 0.63	y = -2.71x +100 R^2 = 0.21	y = -3.52x +100 R^2 = 0.40	y = -3.86x +100 R^2 = 0.48	y = -4.31x +100 R^2 = 0.54	y = -4.96x +100 R^2 = 0.57	y = -0.77x +100 R^2=0.60	y = - 6.67x +100 R^2 = 0.61	y = -7.76x +100 R^2=0.61
Sensitive	y = -0.002x +100 R^2 = 0.83	y= - 3.26x +102 R^2 = 0.60	y = -4.41x +100 R^2 = 0.68	y = -4.91x +100 R^2 = 0.72	y = -5.54x +100 R^2 = 0.74	y = -6.41x +100 R^2 = 0.76	y = -7.48x +100 R^2=0.78	y = -8.67x +100 R^2 = 0.78	y = -10.1x +100 R^2=0.77
Resistant	y = -0.001x +100 R^2 = 0.65	y = -2.08x +100 R^2 = 0.22	y = -2.54x +100 R^2 = 0.42	y = -2.74x +100 R^2 = 0.51	y = -3.02x +100 R^2 = 0.57	y = -3.45x +100 R^2 = 0.60	y = -3.99x +100 R^2=0.62	y = -4.61x +100 R^2 = 0.63	y = -5.36x +100 R^2=0.64

Table 4. Coefficients of determination (R^2) and equations for linear regressions of relative yield versus AOT40 or accumulated ozone stomatal flux ($AF_{st}Y$, mmol m^{-2}) employing a range of flux thresholds (*Y*, nmol m^{-2} s^{-1}). Analyses based on entire dataset (all 15 cultivars); 'Sensitive' cultivars: Claire, Consort, Deben, Korweta, Rialto and Steadfast. 'Resistant' cultivars: Hereward, Kamila, Kobra, Riband, Soissons and Spark. All regressions were significant at the 0.1% level. AOT40: accumulated hourly mean ozone exposure over a threshold of 40 ppb during daylight hours (> 50 W m^{-2}) between 10th May and 14th July; synonymous with the period employed to calculate stomatal ozone flux.

AOT40 and $AF_{st}Y$ indices appeared roughly equivalent in terms of explaining the variance in ozone impacts on yield and yield components (Fig. 2). One contributor to this finding is the confounding influence of the variation between cultivars in measured g_{max} (Table 2). To highlight the potential significance of cultivar variation in g_{max}, Figure 5 shows cumulative modelled O_3 uptake over the course of the season employing extremes in g_{max} recorded in the present study for UK winter wheat cultivars as well as for the extremes taken from the peer-reviewed literature. This approach reveals potential variation in estimated O_3 uptake in the order 32% between UK cultivars, and 79% if extremes reported in the literature are employed for O_3 flux estimation.

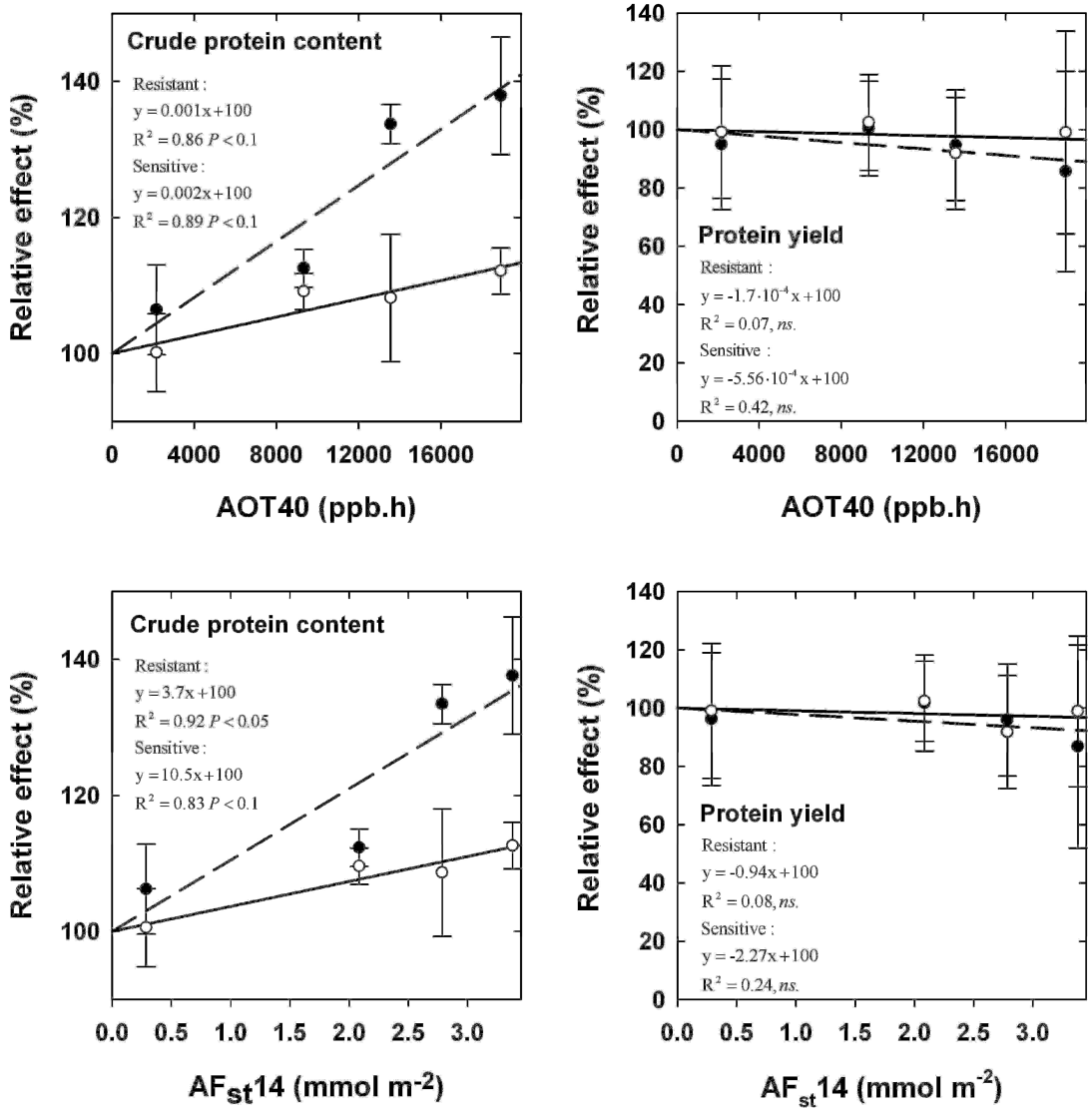

Figure 3. Ozone exposure and dose effects on grain protein (crude protein content and protein yield per ear) of winter wheat fumigated with ozone in open-top chambers. Ozone exposure expressed as AOT40 (ppb.h), accumulated ozone exposure over a threshold of 40 ppb. Ozone dose expressed as accumulated stomatal flux of ozone above a threshold of 14 nmol m^{-2} s^{-1} ($AF_{st}14$ (mmol m^{-2}). Values are expressed in a relative scale. Filled circles represent the sensitive genotype Rialto; open circles represent the resistant genotype Hereward. Dashed line is the linear regression for the sensitive genotype and solid line is the linear regression for the resistant one. Equations, R^2 and P-values of the regressions are also presented.

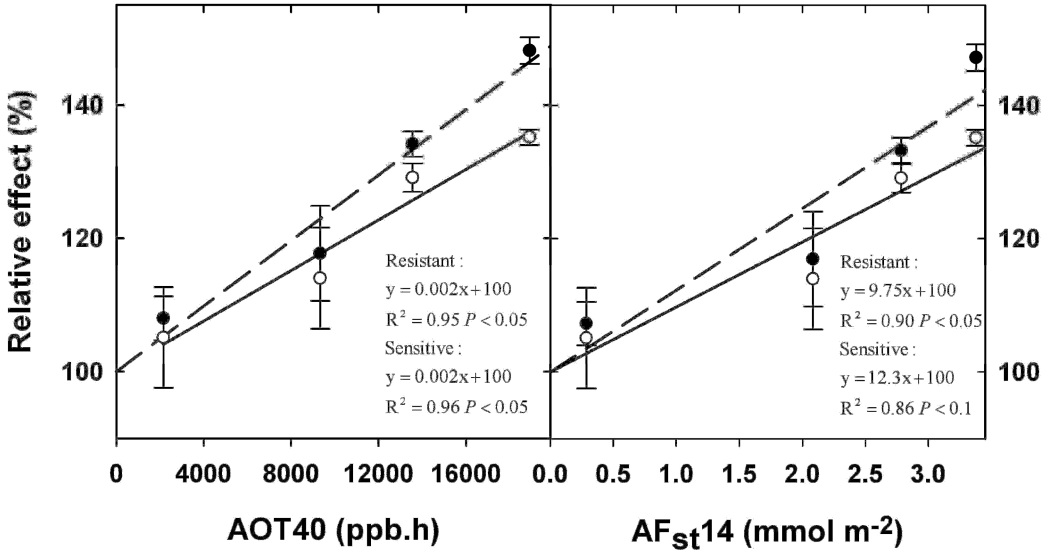

Figure 4. Ozone exposure and ozone dose effects on alpha-amylase activity of winter wheat fumigated with ozone in open-top chambers. Ozone exposure expressed as AOT40 (ppb.h), accumulated ozone exposure over a threshold of 40 ppb. Ozone dose expressed as accumulated stomatal flux of ozone above a threshold of 14 nmol m^{-2} s^{-1} ($AF_{st}14$ (mmol m^{-2}). Values are expressed in a relative scale. Filled circles represent the sensitive genotype Rialto; open circles represent the resistant genotype Hereward. Dashed line is the linear regression for the sensitive genotype and solid line is the linear regression for the resistant one. Equations, R^2 and P-values of the regressions are also presented.

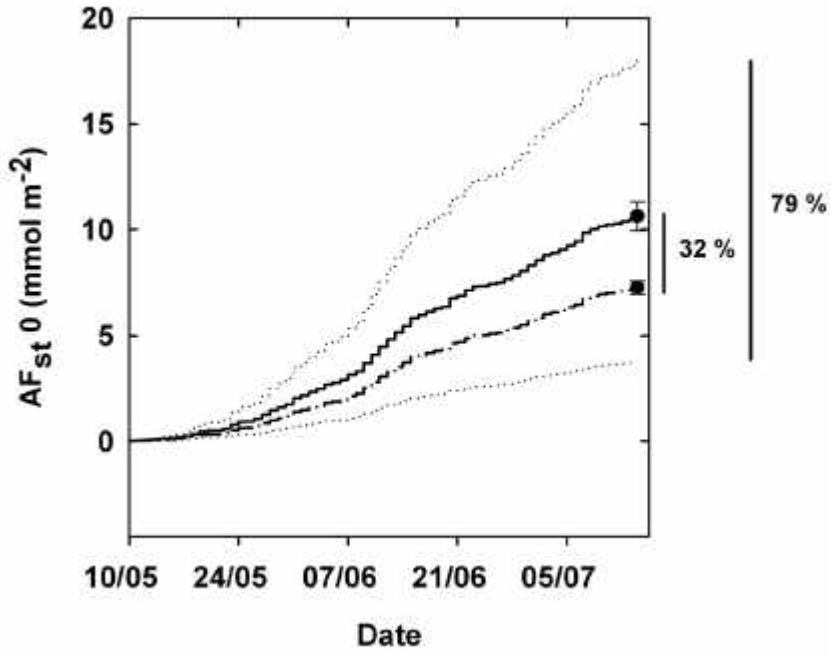

Figure 5. Effect of the measured g_{max} UK variability in stomatal ozone accumulated flux ($AF_{st}0$) (mmol m^{-2}). The bold solid line represents the $AF_{st}0$ using a g_{max} of 649 $\pm$ 31 mmol O_3 m^{-2} PLA s^{-1} in the multiplicative model. The bold dashed-dotted line represents the $AF_{st}0$ if a g_{max} of 434 $\pm$ 18 mmol O_3 m^{-2} PLA s^{-1} is used. Dotted lines represent the variation found on $AF_{st}0$ using the world wide g_{max} variability found in a revision of the literature (803 – 153 mmol O_3 m^{-2} PLA s^{-1}). Final values of $AF_{st}0$ (mmol m^{-2}) are presented as circles with their estimated standard errors (according to the standard errors measured for g_{max}).

4. Discussion

4.1 Stomatal flux model for winter wheat

Table 1 illustrates the close agreement in the boundary lines for PAR, T and VPD between the current g_{H2O} model derived from data for spring wheat (UNECE, 2004) and the measurements made on winter wheat in the present study. However, substantial differences were evident in f_{O3} functions; with winter wheat appearing less responsive to increases in $AF_{st}0$ values than spring wheat. This conclusion is generally consistent with the reported differences in stomatal response to ozone between spring and winter wheat (see review by Feng *et al.*, 2008). There were also marked differences between spring and winter wheat in the f_{phen} function. State-of the-art spring wheat models incorporate a 'plateau' to simulate the high g_{H2O} values recorded after anthesis (Frederick, 1997), but this clouds genotypic variability in the timing of anthesis. Integration of the f_{phen} relationship for winter wheat revealed that this function may account for upto a 12% shift in g_{H2O} predictions. Also, a function representing the influence of time of day on relative g_{H20} (f_{time}) was included in our winter wheat model to simulate circadian changes in g_{H20} (*sensu* Danielson *et al.*, 2003). This approach was adopted in preference to the ? VPD function used in the DO_3SE model (Pleijel *et al.*, 2007) as adoption of the ? VPD function was found to reduce the R^2 of predicted *versus* measured g_{H2O}. This possibly reflects the fact that non-limiting VPD values (0.6 kPa on average) were maintained throughout the experiment and plants were kept well-watered throughout.

Our model tended to overestimate g_{H2O} (see Fig. 1) a frequently encountered situation employing a multiplicative approach - with different parameterizations yielding R^2 values typically ranging between 0.23 and 0.59 (Danielsson *et al.*, 2003; Büker *et al.*, 2007). In the present study, 55% of the hourly variability of g_{H2O} was explained by the derived model, and upto 93% of the mean diurnal variation in g_{H2O}. One confounding factor maybe the high variability in g_{H2O} measurements which is at least partly attributable to genetic variation in g_{max} (Table 2). g_{max} constitutes the cornerstone of Jarvis-style multiplicative modelling approaches for the prediction of g_{H2O} (Emberson *et al.*, 2000; Tuovinen *et al.*, 2004). A critical analysis of the available literature (reviewed in Table 3) revealed no difference in g_{max} between spring and winter wheat. But, significant variability was observed in this study (Table 2) and the extent of the variation (39%) is consistent with that for other studies reporting data for multiple

genotypes e.g. 58% variation was reported in Chinese varieties (Jiang *et al.*, 2000) and 21% in US varieties (Xue *et al.*, 2002). The variability in g_{max} would be considerably greater (c.79%) if the same multiplicative models were to be adopted worldwide. The reduced predictive capabilities of the model due to genetic/regional variation in g_{max} highlight a need to make regional model paramaterisations to improve local ozone risk assessment procedures.

4.2 Genotypic variability of O_3 effects on grain yield and quality-related characteristics

The threshold AOT40 at which the first statistically significant adverse effects on yield were encountered in winter wheat was 3888 ppb.h, which compares favourably with the threshold for effects derived from spring wheat (3000 ppb.h). The highest ozone exposure employed in the present study (NFA+75, 18918 ppb.h) resulted in an average loss in wheat yield of 25%. This compares favourably with the available literature which suggests a 7 h day^{-1} seasonal average O_3 exposure of 42 ppb results in a loss in spring wheat yield of 10-20% (Feng *et al.*, 2008). Genotypes showed considerable variation in their response to the same ozone exposure (i.e. there was evidence of considerable intraspecific variation in 'O_3 sensitivity'; losses in yield varying between 17 and 32% in response to an AOT40 of 18918 ppb.h. The genetic basis for this variation is poorly understood, but such findings suggest there maybe scope for breeders to select genotypes with enhanced ozone tolerance and thus enhanced production capacities in polluted regions (Quarrie *et al.*, 2006). Whilst several studies have focussed on the impacts of ozone pollution on wheat yield, far less attention has been paid to effects on grain quality, and no Critical Levels or Critical Fluxes currently consider effects on crop quality alongside productivity (see Gonzalez-Fernandez *et al.*, 2008). In wheat trading, GPC and a-amylase activity are frequently considered as quality grading factors, determining the end use and price of the grain (Lawlor & Mitchell, 2001). However, scant attention has been paid to O_3 effects on grain quality aspects (Piikki *et al.*, 2008). In this study, O_3 exposure increased GPC and a-amylase activity (Figs. 3 and 4) and although intraspecific variability in responses to the pollutant were evident, the effects observed improving grain quality in elevated ozone would partially offset the negative effects on yield in terms of revenue generation. This conclusion is generally consistent with other reports in the literature (Piikki *et al.*, 2008).

AOT40[4] and the AF_{st} (derived using a threshold of 14 nmol m^{-2} s^{-1}) performed equally well, yielding similar R^2 for all the relationships (Figs. 2-4 and Table 4). Previous studies for spring wheat suggest that flux-based indices out-perform AOT40 across experiments (Pleijel *et al.*, 2007). But this has not always proven to be the case. For example, Karlsson *et al.* (2004) combined data for tree species and found AOT40 to perform slightly better than cumulative flux in predicting effects. This possibly reflects uncertainties in the parameterization of the underlying model of stomatal conductance (Ashmore, 2005). For instance, g_{max} has a strong influence on the modelled $AF_{st}Y$ values (Fig. 5) used to derive dose-response relationships. Moreover, the best performing flux index used a higher cut-off threshold than that usually employed in the derivation of flux-response relationships for spring wheat (Danielsson *et al.*, 2003; Pleijel *et al.*, 2007). However, the O_3 concentration contributing to the accumulated flux at maximum stomatal conductance values was similar, at 20 ppb using threshold values of 5 nmol O_3 m^{-2} PLA s^{-1} (Danielsson *et al.*, 2003). This disparity between models could be the result of differences in g_{max} values and/or a higher intrinsic defense capability in winter versus spring wheat – a view supported by the difference in f_{O3} function for spring (Pleijel *et al*., 2007) and winter (Table 1) wheat.

4.3 Implications for pan-European modelling approaches

Despite the uncertainties described elsewhere (Musselman *et al.*, 2006), the advantages of the flux-based approach to risk assessment is clear. It constitutes a physiologically-relevant representation of the phenology of the crop (Pleijel *et al.*, 2007), takes account of the year-to-year variation in environmental conditions that may affect the period of maximum O_3 sensitivity (Pleijel *et al.*, 1998) and considers environmental variation between sites. However, current models are derived from data for spring wheat. The data shown in this paper indicate a need to consider differences between spring and winter wheat in the sensitivity of g_{H2O} to AFst0, the f_{phen} function and flux thresholds, in order to better qualify the impacts of ozone on wheat at a pan-European level. Moreover, the genetic variation observed in g_{max} and O_3 sensitivity in the present study highlights uncertainties for the development of ozone risk assessment exercises at the European and worldwide scale, and illustrates a need for the development of regionalized risk assessment approaches applicable to local crops and conditions.

[4] considered in EU O_3 pollution regulations (Directive 2008/50/EC)

Acknowledgements

The authors thank Robert Hodgson and Alan Craig for their technical assistance (Newcastle University). Also Benjamín S. Gimeno (CIEMAT, Spain), Andrzej Skoczowski (Polish Academy of Science, Poland) and Paul Bilsborrow (Newcastle University, UK) for their inputs. The study was funded by a combination of EU Framework 5 award (HPMT-CT-2001-00219) and a grant from the Spanish Ministerio de Medio Ambiente, Medio Rural y Marino, "Critical Loads and Levels" (administered via CIEMAT).

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Appendix 1

List of references used in meta-analysis of wheat g_{max}:

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4. IMPACTOS DE LA CONTAMINACIÓN POR OZONO SOBRE LA PRODUCCIÓN Y LA CALIDAD DE PASTOS FORMADOS POR GRAMÍNEAS Y TRÉBOLES

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.

Resumen

Este estudio explora los efectos del ozono (O_3) sobre la productividad y la calidad nutritiva de pastos formados por mezclas de gramíneas y tréboles. También se estudian las interacciones del O_3 con el aporte de nutrientes del suelo y la composición del dosel. Los mesocosmos con mezclas de raigrás y trébol blanco o monocultivos de una de las dos especies, recibieron dos tipos de aporte de nutrientes minerales. La exposición al ozono se realizó en cámaras de techo descubierto, utilizando tres réplicas por tratamiento. Cada tratamiento de ozono expuso las plantas a una concentración acumulada por encima de 40 ppb durante horas diurnas (AOT40) de 60, 3900, 9450 y 17160 ppb.h. Los efectos sobre la productividad se evaluaron cada 28 días durante todo el experimento, mientras que los efectos sobre la calidad del pasto se evaluaron sólo al final.

La exposición al ozono disminuyó la producción y la calidad nutritiva del trébol. Este hecho fue reflejado en el parámetro de CFV (*Consumable Food Value*). Sin embargo, no se encontraron efectos negativos del ozono sobre la producción de raigrás y tan sólo una reducción muy pequeña en la calidad nutritiva. En las mezclas, la proporción de tréboles disminuyó por efecto del ozono. Estas pérdidas fueron compensadas por un aumento en la producción de la gramínea, por lo que ni la productividad ni la calidad nutritiva final de las mezclas se vieron afectada. Los efectos negativos del ozono sobre la productividad del trébol se vieron mitigados cuando éste se encontraba mezclado con raigrás en comparación con los monocultivos.

La dosis acumulada de ozono por encima de un umbral de 8 nmol m^{-2} s^{-1} fue el índice de ozono que mostró el mejor resultado para predecir los efectos del ozono sobre el CFV, en comparación con el AOT40.

Atmospheric Environment 42 (2008) 8755–8769

Contents lists available at ScienceDirect

Atmospheric Environment

journal homepage: www.elsevier.com/locate/atmosenv

Impacts of ozone pollution on productivity and forage quality of grass/clover swards

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ARTICLE INFO

Article history:
Received 16 May 2008
Received in revised form 16 August 2008
Accepted 20 August 2008

Keywords:
Soil nutrition
Ozone risk assessment
Critical levels
Consumable food value

ABSTRACT

This study explored the influence of ozone and interactions with soil nutrient regime and composition on the productivity and nutritive quality of ryegrass/clover swards. Established mesocosms containing ryegrass and/or white clover were grown at two levels of soil nutrition. Mesocosms were exposed in three (replicate) open-top chambers per treatment to accumulated ozone concentrations above 40 ppb during daylight hours (AOT40) of 60, 3900, 9450 and 17,160 ppb h. Effects on productivity were determined at 28-day intervals over the course of the experiment, and nutritive quality was determined for late-season forage.

Ozone exposure was inversely related to clover productivity and nutritive quality, and this was reflected in a similar relationship for consumable food value (CFV). There was no effect of ozone on the productivity of ryegrass and a marginal effect on nutritive quality. In ryegrass/clover mixtures, the clover component was diminished by ozone, and the gaps created were populated by ryegrass with the result that there was no significant effect of ozone on productivity or nutritive quality of ryegrass/clover mixtures. Interestingly, adverse effects of ozone on clover productivity were mitigated in mixtures compared with monocultures.

Accumulated stomatal ozone uptake above a threshold of 8 nmol m^{-2} s^{-1} was found to out-perform AOT40 as a predictor of the effects of ozone on the CFV.

1. Introduction

Air pollution abatement policies have, and are expected to continue to result in a reduction in peak O_3 concentrations across Western Europe and North America (Ashmore, 2005). However, background levels of ozone pollution are rising steadily in the Northern hemisphere (Collins et al., 2000; Vingarzan, 2004). This is a cause for concern, since current ambient levels of ozone (O_3) are known to be high enough to depress crop yields as well as to cause significant changes in the composition and diversity of natural and semi-natural plant communities (Davison and Barnes, 1998; Barnes et al., 1999a,b; Krupa et al., 2004; Bassin et al., 2007).

Considerable attention has been paid to the impacts of O_3 on the yield and floristic composition of grass/clover-dominated grasslands (Nussbaum et al., 1995; Pleijel et al., 1996; Krupa et al., 2004). These studies reveal the clover component to be particularly sensitive to ozone – though

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doi:10.1016/j.atmosenv.2008.08.030

considerable variation may exist both within and between populations of the same species (Barnes et al., 1999a,b). As a consequence, long-term exposure of short-term highly productive leys and semi-natural grasslands to environmentally relevant levels of ozone is commonly reported to result in shifts in species composition; 'resistant' grasses benefitting at the expense of 'sensitive' legumes and forbs (Davison and Barnes, 1998; Ashmore, 2005). In an agricultural context the loss of clover is concerning since this element of the sward constitutes a disproportionately important nutritional resource for ruminant herbivores (Frame, 1987). In managed pastures, grass/clover composition is unstable, with the grass component commonly increasing at the detriment of the legume component over the course of a few years. This occurs at a rate that is influenced by management practices (in particular, cutting and fertilization regimes), early spring temperatures, frost, soil moisture deficit, pests, diseases (Haycock, 1981; Davison and Barnes, 1998) and also the frequency and duration of ozone episodes (Bassin et al., 2007). To date, research conducted by the global air pollution research community has focussed on grassland productivity, with less attention paid to effects on forage nutritive quality (Fuhrer and Booker, 2003; Bender et al., 2006). The few studies that have addressed the effects of ambient, and rising levels of ozone on pasture communities reveal significant reductions in forage nutritive quality – a factor critical for animal production and commercially important, in combination with yield losses, as a driver of meat, milk and fibre production (Krupa et al., 2004; Muntifering et al., 2006). Forage nutritive quality is determined to a considerable extent by cell–wall constituents that are partially and variably digestible, and these fractions can be separated by sequential extraction methods (Van Soest, 1994) that remove soluble cell constituents (including non-structural carbohydrates, lipids, pectin, soluble protein and nonprotein-nitrogen) and isolate insoluble fractions comprising total cell–wall constituents (neutral detergent fibre [NDF]), lignocellulose and protein bound to cell walls (acid detergent fibre [ADF]), lignin and other recalcitrant materials (acid detergent lignin [ADL]). The NDF fraction is inversely related to free-range voluntary forage intake, and the ADF fraction is inversely related to forage digestibility (Van Soest, 1994). To date, the effects of environmentally relevant levels of ozone on consumable food value, an index that integrates effects on grassland productivity and forage quality, have not previously been studied and are thus not incorporated in ozone risk assessment exercises aimed at assessing impacts and establishing critical levels for the protection of grasslands. The improvement of critical levels for the protection of vegetation, which underpin European air pollution abatement legislation, requires the evolution of indices that better explain ozone impacts on relevant plant traits (Fuhrer et al., 1997). There is also a need for improved understanding of the manner in which soil nutrient status influences plant responses to ozone pollution. In some studies, nutrient-rich conditions have been reported to suppress ozone sensitivity (Whitfield et al., 1998), whilst others report no interactive effects (Cardoso and Barnes, 2001) or enhanced sensitivity to the pollutant (Pell et al., 1990). In the present study, we compare exposure–response and dose–response relationships derived from data obtained during an extensive open-top chamber study on established grass/clover mesocosms, with the aim of identifying the most appropriate ozone index upon which to base risk assessment approaches. Early O_3 risk assessment approaches have been based on exposure–response data. However, the advantages associated with dose-based indices, rather than concentration-based indices, are recognized since this approach accounts for the influence of environmental and plant-specific variables on O_3 uptake (Fuhrer et al., 1997; Ashmore, 2005; Fiscus et al., 2005; Crous et al., 2006). Consequently, exposure–response relationships cannot be used to compare impacts of ozone at different locations or from year-to-year, whilst the new generation of flux-based risk assessment approaches provide a more realistic representation of potential impacts, providing a sounder basis for the development of regional air pollution policies (Ashmore, 2005). In order to relate measured effects with stomatal ozone flux, a multiplicative stomatal conductance (g_{H_2O}) model had to be developed for white clover. Herein, we explore the impacts of environmentally relevant levels of ozone on sward productivity and nutritive value and examine interactive effects of soil nutrition and community structure.

2. Materials and methods

2.1. Plant culture and ozone fumigation

Sterilized low nutrient soil was diluted with sand (3 parts soil:1 part sand) and 36 dm^3 was placed in each of 216 rectangular containers ($40 \times 30 \times 30$ cm) and left to over-winter, exposed to the natural environment. In spring of 1999, these 'mesocosms' were randomly allocated to two fertilization regimes ('low nutrition' and 'high nutrition') and perennial ryegrass *Lolium perenne* L. cv. Contender was sown at a rate of 500 kg ha^{-1} into 72 mesocosms allocated to each of these nutrition regimes (i.e. 144 in total). 'Low nutrient' mesocosms received a single fertilizer application at the beginning of each season, a complete liquid fertilizer (Chempak Formula 3; 20N:20P:20K) was applied to the remainder ('high nutrient' mesocosms) once per month at a dose of 500 ml (1 g) per m^2 of soil. All mesocosms were cultured outside and plants allowed to establish for the remainder of the year – with cuts undertaken at 28-day intervals throughout the year.

In February of 2000, virus-free single-node cuttings of 'NC-S' white clover (Heagle et al., 1995) (*Trifolium repens* L. cv. Regal clone NC-S; supplied by the co-ordination centre of the UNECE-ICP Vegetation Programme at CEH Bangor, Gwynedd, Wales) were grown in potting compost (John Innes No. 2 potting compost, a general plant compost comprising 7:3:2 parts (per volume) of loam, peat and sand, respectively) in a glasshouse and, in May, using a standard template, six rooted-plants were transplanted in to 72 of the ryegrass mesocosms (to create 36 'low nutrient' and 36 'high nutrient' mixed mesocosms) plus 72 mesocosms without ryegrass (to create 36 'low nutrient' and 36 'high nutrient' clover monocultures). NC-S white clover was chosen for the present study because of the wealth of

background information on this genotype as well as the availability of virus-tested stock plants. The clonal stock originates from a selection of ozone-sensitive genotypes made in the field in North Carolina, U.S. (Heagle et al., 1994). The genotype has been widely employed for biomonitoring studies across Europe and North America, and constitutes the current cornerstone of UNECE-ICP Vegetation ozone biomonitoring programmes (Heagle et al., 1995; Mills et al., 2002).

Following establishment, mesocosms were transferred (in late-June) to one of twelve rigid open-top chambers (OTCs) comprising part of a field fumigation facility situated at Newcastle University's Close House Field Station at Heddon-on-the-Wall, Northumberland (National Grid Reference NZ 128659, latitude 54°59′ N, longitude 1°48′ W, elevation 30 m.a.s.l.). Each chamber, octagonal in shape (3.5 m max diameter by 3.3 m in height), was constructed from an aluminium framework, clad with standard horticultural glass, with a plenum incorporated just below the mouth of the chamber (at 3.1 m from ground-level) to reduce incursion of ambient air (see Crookshanks et al., 1998). The area occupied by each chamber was dug-out so that every chamber was sited on a level gravel-chipped base divided in three blocks. Mesocosms were randomized within the three blocks in each OTC in such a manner that three pots of each combination were represented in every block. Air was supplied to each chamber by a free-standing fan unit housing a particulate filter, with the air introduced into each chamber through PVC ducting via a 'doughnut' made from 140 gauge polyethylene tubing (containing approximately 280 × 25 mm holes) positioned 1.5 m above ground-level. A programmable PC-based time-share ozone control/logging system was used to monitor, adjust and graph the ozone level in individual chambers, based on hourly records/adjustments for every chamber. The control system adopted is described in detail elsewhere (Barnes et al., 1995), the only modification for this application being the adoption of a geared motor to regulate the voltage output of the ozone generator to control O_3 delivery. All OTCs were ventilated with non-filtered air (NFA) at a rate sufficient to achieve 2 air changes min^{-1} in each chamber. Ozone treatments were randomly assigned to 12 open-top chambers and, on 4th of August 2000, ozone was injected into the NFA stream supplying nine of the chambers to attain four target ozone treatments 7 h d^{-1} in three (replicate) OTC per treatment: NFA, NFA + 25, NFA + 40 and NFA + 55 ppb[1] O_3. Ozone was generated from oxygen by electric discharge (Model SCA01 Pacific Ozone Technology Inc., El Sobrante, California, USA) and regulated on the basis of measurements made at canopy height in the centre of each OTC with a photometric analyser (Dasibi, Model 1008 UV Photometric Ozone Analyzer). The ozone analyser was serviced weekly and calibrated monthly. Calibration was performed against a Dasibi 1008PC unit, in-turn calibrated at 6-monthly intervals against National Physics Laboratory standards. The level of ozone in individual chambers was checked against target levels daily, by an on-site instrumentation technician, and where minor adjustments were required. The introduction of ozone to each chamber was manually regulated via stainless steel gap flow meters equipped with a needle valve.

2.2. Stomatal conductance measurements

Abaxial stomatal conductance measurements were made on fully expanded leaves of clover over a range of environmental conditions using a Delta-T AP4 Porometer (Delta-T Devices, Ltd, Cambridge, UK). Measurements were restricted to leaves at the top of the canopy of well-fertilized monocultures, and were performed during the second 28-day regrowth period (i.e. harvest 2 interval, 23rd August to 17th October). Photosynthetically active radiation (PAR) and leaf temperature (T_L) were recorded at the time of each measurement. Due to problems with on-site equipment, relative humidity (RH) was obtained from a Meteorological Office monitoring station (Newcastle Weather Centre; altitude 52 m.a.s.l; latitude 54°98′ N, longitude 1°60′ W) situated 18 km from the experimental site. Relative humidity (RH) was used to calculate the vapour pressure deficit (VPD) according to Goff and Gratch (1946).

2.3. Productivity and forage quality

Mesocosms were cut to leave 5 cm of above-ground stubble on transfer to OTCs, and this procedure was repeated at 28-day intervals. The first harvest, where material was retained, was performed on 31st August and the third was on 26th October 2000. Harvested plant material was dried to constant weight in an oven at 50 °C and dry matter yield determined for grass and clover separately in order to determine impacts on regrowth in a species-specific manner in mixtures. Chemical analyses were performed on plant material recovered at the third harvest.

Dried plant material was ground in a mill to pass a 1 mm screen. Random sub-samples representative of grass and clover from both low and high nutrition treatments (three samples per ozone level) were employed for C/N analysis (Carlo Erba Instrument) and element profiling using Inductively Coupled Plasma analysis.

Neutral detergent (NDF) and acid detergent (ADF) fractions plus acid detergent lignin (ADL) were determined sequentially, following the procedures described by Van Soest et al. (1991). Samples for nutritive analyses from mixed mesocosms did not differentiate between species. In vitro dry matter digestibility (IVDMD) was determined by Goering and Van Soest (1970) modification of the two-stage Tilley and Terry (1963) procedure, and was restricted to clover monocultures. Parameters relating forage quality to animal performance, such as percentage digestible dry matter (%DDM) (Linn and Martin, 1989), dry matter intake (DMI) predicted from NDF (Mertens, 1987) and non-digestible fibre from IVDMD (Goering and Van Soest, 1970) were determined. Relative food value (RFV, Rohweder et al., 1978) was calculated by reference to a digestible DM intake that has been adopted to standardize a forage containing 53% NDF and 41% ADF to a RFV of 100 (Linn and Martin, 1989). Consumable food value (CFV) was also calculated to integrate impacts of ozone, nutrition and/or sward

[1] 1 part per billion v/v (ppb) = 1 nmol mol^{-1}.

composition on forage feed value in terms of productivity and nutritional quality (sensu Krupa et al., 2004). Equations used to calculate these parameters are provided in a appendix to the manuscript. Ruminal fluid used for IVDMD determinations was obtained 3 h postprandial from a mature, ruminally fistulated dairy cow (*Bos taurus*) fed grass hay *ad libitum*. Animal maintenance, experimental protocols, and all surgical procedures plus post-surgical care were reviewed and approved by the Auburn University Institutional Animal Care and Use Committee (Protocol Review Number 0207-R-2168).

2.4. Stomatal conductance model

A multiplicative approach to modeling stomatal conductance was adopted (sensu Jarvis, 1976). Using this approach, stomatal conductance (g_{H_2O}) is predicted from a multiplicative function describing the way in which key drivers (including PAR, VPD, T, time of day and leaf age) influence g_{H_2O} under the assumption that specific drivers act in an independent manner. The impacts of key environmental and intrinsic variables on g_{H_2O} were determined using boundary line analysis following the methodology described in Schmidt et al. (2000). The maximum g_{H_2O} (g_{max}), measured under the prevailing experimental conditions, was calculated as the average of the recorded values above the 90th percentile of the entire data set. Minimum g_{H_2O} was calculated similarly using the average of the g_{H_2O} values below the 10th percentile. Plants were kept well-watered, thus soil moisture deficit (SMD) was considered not to influence g_{H_2O}. A factor allowing the correction of modelled g_{H_2O} for the influence of ozone uptake on g_{H_2O} was calculated and used in the derivation of dose–response relationships (Pleijel et al., 2007). To evaluate the derived models, predicted values were compared with measured data using r^2, p-values resulting from linear regressions and the root-mean-square scaled deviation (RMSSD), which measures the absolute deviation between modelled and experimental values (Schunn and Wallach, 2001). Comparison with a perfect fit (i.e. where $x = y$) between modelled and measured data was achieved using the 95% confidence intervals of the linear regression coefficients.

2.5. Ozone indices

The relationship between yield or quality-related parameters and ozone exposure was assessed through the use of the AOT*X* (i.e. the accumulated exposure to ozone over a given threshold (*X*, ppb)) during daylight hours (Fuhrer et al., 1997). Accumulated stomatal ozone flux above a critical threshold ($AF_{st}Y$, where Y is the threshold applied in nmol m^{-2} s^{-1}) was calculated (see appendix) according to the principles outlined in the Mapping Manual of the LRTAP Convention (UNECE, 2004). Different flux thresholds were applied (ranging from 0 to 60 ppb, 10 ppb stepwise variation in the exposure index and from 0 to 12 nmol m^{-2} s^{-1}, at 2 nmol m^{-2} s^{-1} variation, when using the dose-based indices), then measured effects on the clover fraction were linearly related with the calculated flux in order to establish exposure– and dose–response relationships, following the methodology outlined by Fuhrer (1994). Linear regressions between the ozone index and the response were performed, then each response value was divided by the intercept of its regression equation, so that zero O_3 exposure was always associated with no effect (i.e. a relative value of 100%).

2.6. Statistical analysis

Statistical analyses were performed using Statistica 6.0 (StatSoft Inc., Tulsa, USA). All data were first checked for normal distribution and homogeneity of variance, using Shapiro–Wilks and Bartlett tests, respectively. Data were transformed when assumptions were not fulfilled. All percentage data were subject to arcsine square root transformation prior to MANOVA. Data were then subjected to analysis of variance (ANOVA) to investigate the influence of chamber on all measured parameters. No significant chamber-to-chamber variation was evident within treatments. Inter-harvest biomass data for each species were reanalysed using multivariate analysis of variance (MANOVA) to test the effects of O_3, nutrition (N) and sward composition (C), under the assumption that plants in replicate chambers were as likely to be as similar to, or as different from, plants within an individual chamber, based on biomass yield in individual chambers at harvests 1, 2 and 3.

Chemical fractions were also analysed using MANOVA (dependent variables: NDF, ADF and ADL; independent variables: O_3, N and sward composition (clover, grass or mixtures)), with detailed nutritive analyses restricted to harvest 3 (H3). Treatment effects on IVDMD were analysed using two-way ANOVA and RFV and CFV with three-way ANOVA. Significant differences between ozone treatments and sward composition were tested using planned (a priori) comparisons and post-hoc Bonferroni tests (calculated at the 5% level).

Linear regressions were employed to relate O_3 exposure, or dose, with their corresponding relative effects. Relative values were calculated using the NFA treatment as a control. When the residuals of the regression showed a non-linear relationship between O_3 index and effect a curve was fitted to the data. If ANOVA revealed no significant interactions in, and overlap between, confidence intervals then responses for individual treatments were combined. Graphs were plotted using SigmaPlot v.9.0 (Systat Software Inc.).

3. Results

3.1. Ozone exposure, climatic conditions and nutritional regimes

Ozone treatments represented AOT40s (accumulated hourly ozone concentrations above a threshold of 40 ppb during daylight hours) of 60, 3900, 9450 and 17,160 ppb h, delivered between 4th August and 26th October (see Fig. 1). Day-time average temperatures decreased from 17 °C at harvest 1 to 11 °C at harvest 3. Similarly, average day-time PAR values decreased from 896 (H1) to 624 (H3) μmol m^{-2} s^{-1}, and VPD from 0.6 (H1) to 0.4 (H3) kPa over the course of the experiment.

Chemical analysis revealed the 'low nutrition' treatment to significantly ($p < 0.01$, d.f. = 68) reduce foliar N, K and Zn

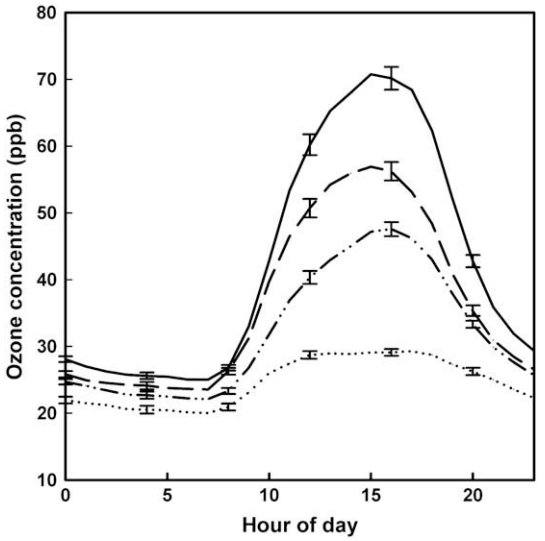

Fig. 1. Hourly average diel ozone concentrations (ppb) over the course of the open-top chamber fumigation. Treatments: Non-filtered air [NFA] (dotted line), NFA + 25 (dash-dotted line), NFA + 40 (dashed line) and NFA + 55 (solid line) over the course of the season. Error bars shown (at four-hour intervals) depict SE over the course of the season.

concentration plus C/N ratio in ryegrass, but there were no significant effects of nutrient regime on the elemental composition of white clover, other than a marginal ($p < 0.1$, d.f. = 68), but consistent, reduction in foliar N, P and K content (Table 1). Ozone treatment had no significant effect on foliar nutrient composition in both species and there was no evidence of any ozone × nutrient regime interactions.

3.2. Impacts of ozone on sward productivity

Table 2 provides a summary of the effects of ozone and interactive effects of soil nutrition and sward composition on the productivity (above-ground dry matter accumulation between 28 d harvest intervals; DW) of ryegrass, white clover and ryegrass/clover mixtures. Biomass accumulation declined significantly ($p < 0.001$, d.f. = 45) from the first to the third harvest. This decline in sward productivity was positively related to changes in average weather conditions over the experimental period; linear regressions using temperature (T) as the independent variable yielded highly significant relationships ($p < 0.001$, $n = 36$; $r^2 = 0.54$, 0.45 and 0.47 for ryegrass, clover and mixtures, respectively). When the depression in biomass production associated with deteriorating weather conditions was removed, ANOVA revealed no significant differences in yield between harvests other than that due to treatment regimes.

MANOVA indicated that ozone, soil nutrient regime and sward composition influenced biomass production. Ryegrass biomass yield was not significantly influenced by O_3 exposure ($p = 0.289$, d.f. = 32). However, clover grown in the same chambers showed a decline in yield from harvest 2 onwards ($p < 0.001$, d.f. = 32 H2 and H3), with effects proportional to ozone exposure. At the highest level of ozone exposure (NFA + 55) there was a 53% reduction in clover biomass production by H3 (relative to NFA). In mixed mesocosms, the clover/ryegrass biomass ratio (i.e. the fractional contribution of clover to the sward) declined as the season progressed (see Fig. 2). Ozone treatment appeared to aggravate shifts in the clover/ryegrass balance, with ozone effects significant in 'low nutrient' mesocosms relative to the control (NFA) ($p = 0.012$, 0.04 and 0.02, d.f. = 14, H1, H2 and H3, respectively), but not in the 'high nutrient' regime. Impacts of ozone on total biomass yield of mixed mesocosms were tempered by the shift in clover/ryegrass contribution, resulting in no significant ozone effect on total biomass yield.

Nutrient regime had a strong influence on sward productivity. Ryegrass monocultures appeared the most affected; 'low nutrient' treatment resulting in a significant ($p < 0.001$, d.f. = 32) depression in yield – 47% (H1), 57% (H2) and 67% (H3) with respect to 'high nutrient' regime. Consistent with observed effects of soil nutrient regimes on plant nutritional status, impacts of the 'low nutrient' treatment on clover monocultures were significant ($p < 0.01$, d.f. = 32), but impacts decreased over the course of the experiment and by the end there was no significant difference in clover productivity between nutrient treatments (H3; $p = 0.164$, d.f. = 32). Yield of mixed mesocosms was also depressed ($p < 0.001$, d.f. = 14) in the 'low nutrient' treatment; by 29, 34 and 44 % at H1, H2 and H3, respectively. Nutrient regime also exerted significant shifts on the species balance in mixed mesocosms with the clover/ryegrass biomass ratio significantly ($p = 0.013$, d.f. = 14) higher in the 'low nutrient' treatment (at all three harvests). This indicates a nutrient-related shift in competitive balance – clover out-competing ryegrass in mesocosms subject to the 'low nutrient' regime. No statistically significant O_3 × soil nutrient interactions were evident ($p = 0.35$, d.f. = 48).

Clover yield in mixed mesocosms was significantly ($p < 0.001$, d.f. = 32) less than that in monocultures, and ozone effects were influenced by sward composition (monoculture versus mixture). At H3, clover biomass growing in mixtures was significantly less affected by ozone than clover biomass in monocultures ($O_3 \times C$ $p = 0.05$, d.f. = 32). In contrast, there was no statistically significant increase in ryegrass yield in mixtures compared with monocultures until H3. No statistically significant soil nutrition × C interactions were evident throughout.

3.3. Impacts on forage quality

Table 3 provides a summary of the effects of ozone and soil nutrition on parameters associated with the nutritive quality of forage for ruminant animals. Ozone fumigation resulted in no significant effects on parameters related to the quality of ryegrass forage, but resulted in significantly elevated concentrations of NDF and ADL ($p = 0.006$, and $p = 0.01$, d.f. = 129 respectively) in clover forage. Similarly, concentration of non-digestible fibre from the IVDMD analysis was significantly ($p < 0.01$, d.f. = 15) increased by ozone exposure. Concentrations of NDF, ADL and non-digestible fibre from IVDMD increased proportionately with exposure to ozone. Forage quality of mixed samples

Table 1
Impacts of ozone and soil nutrient regime on foliar nutrient composition of clover and ryegrass

Ozone	Nutrient	Spp	Foliar nutrient composition											
			C[a]	N[a]	K[a]	Mg[a]	Ca[a]	P[a]	S[a]	Cu[b]	Zn[b]	Fe[b]	Mn[b]	C/N
NFA	LN	Ryegrass	409	22.8	3.1	2.6	7.7	3.6	422	1.5	39	46.4	301	17.9
		Clover	419	40.5	2.4	1.9	1.9	3.4	215	2.7	43	67.7	161	10.3
	HN	Ryegrass	417	25.0	3.7	2.6	6.0	4.3	404	1.4	42	43.0	169	16.7
		Clover	413	41.1	2.6	1.9	1.7	4.0	212	1.6	38	64.5	135	10.0
NFA25	LN	Ryegrass	404	19.4	2.7	2.1	7.8	3.5	377	2.0	48	88.7	374	20.8
		Clover	424	42.3	1.5	1.7	1.6	3.5	211	1.5	39	44.1	92	10.0
	HN	Ryegrass	416	25.8	3.7	2.7	6.3	4.3	394	1.5	45	53.6	190	16.1
		Clover	424	43.2	2.6	1.9	1.7	3.9	203	1.4	42	48.7	193	9.8
NFA40	LN	Ryegrass	411	23.8	3.2	2.6	7.8	4.4	460	2.0	40	71.4	242	17.3
		Clover	417	41.6	2.1	1.9	1.9	3.5	210	1.7	39	54.5	107	10.0
	HN	Ryegrass	420	26.3	3.8	2.6	5.9	4.5	429	2.2	49	132.0	155	16.0
		Clover	420	42.2	2.4	1.9	1.7	3.9	204	1.6	39	60.1	105	10.0
NFA55	LN	Ryegrass	410	21.3	3.0	2.4	7.7	3.8	421	2.1	48	79.4	275	19.2
		Clover	414	40.4	2.4	1.9	1.9	3.3	214	5.4	53	17.6	119	10.2
	HN	Ryegrass	417	25.6	3.8	2.6	6.3	4.4	404	1.2	37	32.9	216	16.3
		Clover	404	41.6	2.7	1.9	1.8	4.0	217	2.3	39	10.8	117	9.7
ANOVA results														
Ryegrass	O_3		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	N		<0.01	<0.001	<0.01	ns	<0.001	ns	ns	ns	<0.001	ns	<0.01	<0.01
	$O_3 \times N$		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Clover	O_3		ns	ns	ns	ns	ns	ns	ns	<0.05	ns	ns	ns	ns
	N		ns	ns	ns	ns	<0.05	ns	ns	ns	ns	ns	<0.001	ns
	$O_3 \times N$		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Mesocosms, grown at two levels of soil nutrition, were exposed in three (replicate) open-top chambers per treatment to four ozone regimes delivering AOT40s of 60 (NFA), 3900 (NFA25), 9450 (NFA40) and 17,160 (NFA55) ppb h. Harvests were made at 28-day intervals.
ns, statistically non-significant results ($p > 0.05$).
[a] Foliar composition in mg g^{-1}.
[b] Foliar composition in μg g^{-1}.

Table 2
Impacts of ozone, soil nutrient regime and sward composition on forage yield (dry weight, g)

HARVEST			HARVEST 1				HARVEST 2				HARVEST 3			
Ozone treatment			NFA	NFA + 25	NFA + 40	NFA + 55	NFA	NFA + 25	NFA + 40	NFA + 55	NFA	NFA + 25	NFA + 40	NFA + 55
MONO	LN	Ryegrass	4.3	4.0	3.6	2.6	1.8	2.7	1.4	2.4	1.2	1.1	0.6	0.8
		Clover	11.6	11.9	14.9	10.7	10.4	10.8	8.9	6.9	5.0	4.4	3.3	2.3
	HN	Ryegrass	7.6	6.6	7.2	6.5	5.7	6.1	6.0	5.1	3.6	3.4	3.2	3.3
		Clover	17.0	13.8	12.3	16.9	13.7	11.4	10.4	8.6	6.0	5.3	3.4	2.5
MIXTURE	LN	Ryegrass	3.2	3.1	3.0	3.0	2.0	3.6	2.2	2.4	1.4	2.0	1.4	1.7
		Clover	10.6	8.1	9.2	5.5	7.2	3.9	7.7	3.5	1.8	1.2	1.5	1.1
		Total	13.7	11.3	12.3	8.7	9.3	7.5	9.9	5.3	3.2	3.2	2.9	2.8
	HN	Ryegrass	5.6	5.2	5.6	6.7	5.0	4.3	5.2	6.2	3.4	4.2	2.9	3.8
		Clover	12.1	10.2	10.0	8.7	9.2	7.3	6.6	4.4	2.6	1.6	1.8	1.3
		Total	17.7	15.4	15.7	15.4	14.2	11.6	11.8	10.6	6.0	5.8	4.6	5.2

MANOVA results	HARVEST 1			HARVEST 2			HARVEST 3		
Univariate tests	Clover[a]	Ryegrass[b]	Mixture	Clover[c]	Ryegrass[d]	Mixture	Clover[e]	Ryegrass[f]	Mixture
O_3	ns	ns	ns	<0.001	ns	ns	<0.001	ns	ns
N	0.005	<0.001	0.001	0.03	<0.001	0.001	ns	<0.001	<0.001
C	<0.001	ns	–	<0.001	ns	–	<0.001	<0.01	–
$O_3 \times N$	ns	ns	ns	ns	ns	ns	ns	ns	ns
$O_3 \times C$	ns	ns	–	ns	ns	–	0.05	ns	–
$N \times C$	ns	ns	–	ns	ns	–	ns	ns	–
$O_3 \times N \times C$	ns	ns	–	ns	ns	–	ns	ns	–

Mesocosms, grown at two level of soil nutrition, were exposed to four ozone regimes delivering AOT40s of 60 (NFA), 3900 (NFA25), 9450 (NFA40) and 17,160 (NFA55) ppb h in three (replicate) open-top chambers per ozone treatment. Harvests were made at 28-day intervals.
LN = 'low nutrient' regime. HN = 'high nutrient' regime. ns, statistically non-significant result ($p > 0.05$). Superscripted letters refer to transformation applied prior to ANOVA.
[a] $y' = (y + 1.69)^{0.15}$.
[b] $y' = (y + 1.69)^{0.15}$.
[c] $y' = (y + 0.92)^{0.4}$.
[d] $y' = (y + 0.92)^{0.67}$.
[e] $y' = (y + 0.34)^{0.43}$.
[f] $y' = (y + 0.34)^{0.43}$.

from ryegrass/clover mesocosms was not significantly affected by ozone fumigation.

The 'low nutrient' regime resulted in a significant ($p < 0.01$, d.f. = 13) increase in ADF concentration in ryegrass forage. In contrast, no significant effects on forage quality-related parameters were detectable in clover. Forage from ryegrass/clover mixtures exhibited increased ($p < 0.01$, d.f. = 15) ADL concentration in the 'low nutrient' regime.

Sward composition significantly ($p < 0.001$, d.f. = 82) affected NDF, ADF and ADL concentrations averaged across ozone fumigation treatments and soil nutrient regimes. Ryegrass monocultures exhibited the highest ($p < 0.001$, d.f. = 43) NDF and ADF values. In contrast, ADL concentration was 64% higher ($p < 0.001$, d.f. = 43) for clover versus ryegrass (monocultures). A significant ($p < 0.05$, d.f. = 43) nutrient regime × C interaction was found for ADL concentration.

3.4. Impacts on relative food value (RFV) and consumable food value (CFV)

Table 4 summarises effects of ozone and nutrition on parameters that reflect forage intake potential and

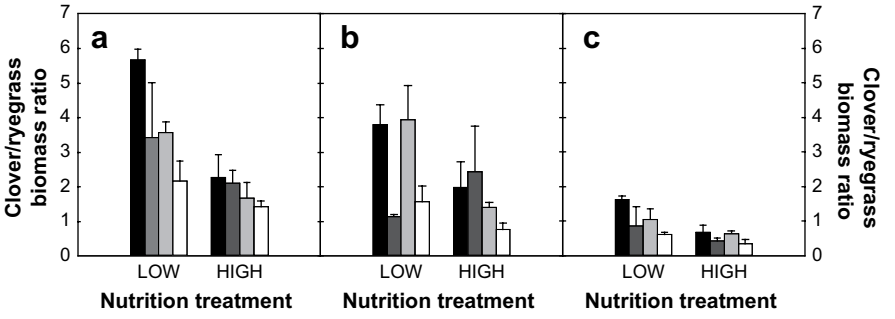

Fig. 2. Impacts of ozone and soil nutrient regime on clover/ryegrass biomass ratio in 'mixed mesocosms' at the three experimental harvests: (a) harvest one; (b) harvest two and; (c) harvest three. Plants were exposed in three (replicate) open-top chambers per treatment to four ozone regimes delivering AOT40s of 60 (NFA) (black bars), 3900 (NFA25) (dark grey bars), 9450 (NFA40) (grey bars) and 17,160 (NFA55) (white bars) ppb h over the course of the season. Error bars represent the standard error of the mean ($n = 3$).

Table 3
Impacts of ozone, soil nutrient regime and sward composition on parameters related to forage nutritive quality value

Ozone treatment		NDF				ADF				ADL				Non-digestible			
		NFA	NFA + 25	NFA + 40	NFA + 55	NFA	NFA + 25	NFA + 40	NFA + 55	NFA	NFA + 25	NFA + 40	NFA + 55	NFA	NFA + 25	NFA + 40	NFA + 55
Low N	Ryegrass	55.9	58.4	59.7	60.0	28.5	30.4	29.1	29.5	1.2	1.7	1.0	1.4	–	–	–	–
	Clover	34.4	34.3	36.6	38.8	22.1	21.8	21.8	24.9	3.4	3.9	4.2	4.8	8.8	9.5	10.4	13.9
	Mixture	43.2	43.3	44.3	46.0	24.2	24.3	23.5	26.1	2.6	1.9	2.8	2.5	–	–	–	–
High N	Ryegrass	51.8	56.0	53.9	56.5	25.5	26.8	24.9	25.5	1.9	2.0	0.3	1.4	–	–	–	–
	Clover	31.7	34.5	35.7	38.4	21.5	20.9	21.9	23.9	3.0	3.6	4.4	4.3	8.8	9.7	11.3	13.0
	Mixture	44.5	45.7	44.0	44.6	24.2	24.4	22.9	23.1	2.2	1.2	1.9	1.5	–	–	–	–

MANOVA results		Univariate test results			
Multivariate test		NDF	ADF	ADL	Non-digestible fibre from IVDMD[a]
O_3	0.02	0.02	ns	ns	0.04
N	0.04	ns	0.007	ns	ns
C	< 0.001	<0.001	<0.001	<0.001	–
$O_3 \times N$	0.73	ns	ns	ns	ns
$O_3 \times C$	0.15	ns	ns	ns	–
$N \times C$	< 0.01	ns	ns	0.03	–
$O_3 \times N \times C$	0.99	ns	ns	ns	–

Mesocosms, grown at two level of soil nutrition, were exposed to four ozone regimes delivering AOT40s of 60 (NFA), 3900 (NFA25), 9450 (NFA40) and 17,160 (NFA55) ppb h in three (replicate) open-top chambers per ozone treatment. NDF, Neutral detergent fraction (%); ADF, Acid detergent fraction (%); ADL, Acid detergent lignin (%); IVDMD, in vitro dry matter digestibility (%).
ns, statistically non-significant result ($p > 0.05$).
[a] IVDMD was only determined for clover plants grown in monoculture.

Table 4
Impacts of ozone, soil nutrient regime and sward composition on parameters related to forage nutritive quality

Ozone treatment		RFV				CFV			
		NFA	NFA + 25	NFA + 40	NFA + 55	NFA	NFA + 25	NFA + 40	NFA + 55
Low N	Ryegrass	112.0	104.0	103.1	102.2	1.2	0.82	0.43	0.52
	Clover	194.7	198.9	183.2	166.6	1.0	0.88	0.62	0.64
	Mixture	150.9	151.1	149.2	138.7	0.99	1.1	0.92	0.8
High N	Ryegrass	124.7	113.3	120.2	113.7	3.3	2.9	2.9	2.8
	Clover	212.3	196.3	187.4	174.1	1.3	1.0	0.67	0.44
	Mixture	146.7	143.5	150.4	148.0	1.8	1.7	1.4	1.6

ANOVA results	RFV	CFV
O_3	0.059	<0.001
N	0.127	<0.001
C	<0.001	<0.001
$O_3 \times N$	0.82	0.96
$O_3 \times C$	0.39	0.58
$N \times C$	0.44	<0.001
$O_3 \times N \times C$	0.97	0.61

Mesocosms, grown at two level of soil nutrition, were exposed to four ozone regimes delivering AOT40s of 60 (NFA), 3900 (NFA25), 9450 (NFA40) and 17,160 (NFA55) ppb h in three (replicate) open-top chambers per ozone treatment. RFV, relative food value; CFV, consumable food value.

digestibility (RFV), and the integration of effects on these with those on forage productivity (CFV). The RFV of clover monocultures was 2.4 times greater ($p < 0.001$, d.f. = 42) than that of equivalent grass mesocosms. Ozone fumigation had no effect on RFV of ryegrass, but significantly ($p < 0.05$, d.f. = 43) reduced RFV of clover forage. No significant interactive effects of soil nutrition or sward composition were detected nor was there any significant effect of ozone on RFV of mixed samples from grass/clover mesocosms.

Interestingly, ozone exposure was found to significantly ($p = 0.01$, d.f. = 43) reduce CFV of ryegrass subject to 'low nutrient' regime, but there was no statistically significant effect in the 'high nutrient' regime. In contrast, ozone fumigation resulted in a marked ($p < 0.001$, d.f. = 43) decline in CFV in both 'low' and 'high' nutrient regimes in clover. In ryegrass/clover mixtures, ozone exposure was found to result in no significant change in CFV. The 'low nutrient' treatment resulted in a marked ($p < 0.001$, d.f. = 43) depression in CFV of both monocultures, with differences between ryegrass and clover strongly dependent on nutrient supply (nutrient regime × sward composition $p < 0.001$, d.f. = 43). There was no significant difference in CFV between ryegrass and clover in the 'low nutrient' treatment, but in the 'high nutrient' regime CFV was three-fold higher for ryegrass than clover (nutrient regime × sward composition $p < 0.001$, d.f. = 43).

3.5. Multiplicative stomatal conductance model

An algorithm describing the manner in which key environmental variables influenced stomatal conductance was derived from a database comprising 258 measurements made across a range of environmental conditions over the lifespan of leaves at the top of the canopy. Measured stomatal conductance data were transformed to values relative to g_{max} and algorithms derived from boundary line analyses with respect to the influence of PAR, *T* and VPD on relative stomatal conductance. Leaf age and time of day were not considered in the final model employed to calculate stomatal ozone flux because the inclusion of equations describing the influence of these parameters on relative stomatal conductance did not improve the relationship between measured and modelled g_{H_2O} values. Consequently, the simplest model, considering PAR, *T*, VPD and a correction factor based on the accumulated ozone flux ($AF_{st}0$) was used to derive dose–response relationships. Fig. 3 reveals that this relatively simple model yielded good agreement between modelled and measured g_{H_2O} values ($r^2 = 0.72$, $n = 20$ and RMSSD = 1.06 employing hourly averages; $r^2 = 0.42$ $n = 258$ and RMSSD = 0.97 employing instantaneous values) and relationships between modelled and predicted data were linear and close to where $x = y$ (hourly averaged g_{H_2O} data: $g_{modelled} = 1.11 g_{measured} = 14.7$, $p < 0.001$, $n = 20$, not significantly different from the line where $x = y$; instantaneous g_{H_2O} data: $g_{modelled} = 0.78 g_{measured} + 161$, $p < 0.001$, $n = 258$, significantly different from the line where $x = y$).

3.6. Exposure–response versus dose–response relationships

The accumulated hourly ozone concentration during daylight hours over a specific threshold (AOT*X*) was employed for the derivation of exposure–response relationships for ryegrass, clover and ryegrass/clover mixtures. AOT thresholds ranging from 0 to 60 ppb O_3 were examined. As the use of alternative AOT thresholds only improved exposure–response relationships by (max.) 3%, the AOT40 is adopted herein. Also cumulative ozone exposure periods were tested, considering only the current growing period or the accumulated AOT40 since the beginning of the fumigation experiment. The latter approach provided the strongest predictor of yield losses.

Exposure–response relationships for ryegrass, clover and ryegrass/clover mixtures were derived considering different thresholds (ranging from 0 to 60 ppb, 10 ppb stepwise variation), using data for replicate chambers averaged across soil nutrient treatments (no evidence of interactive effects was detectable and confidence intervals

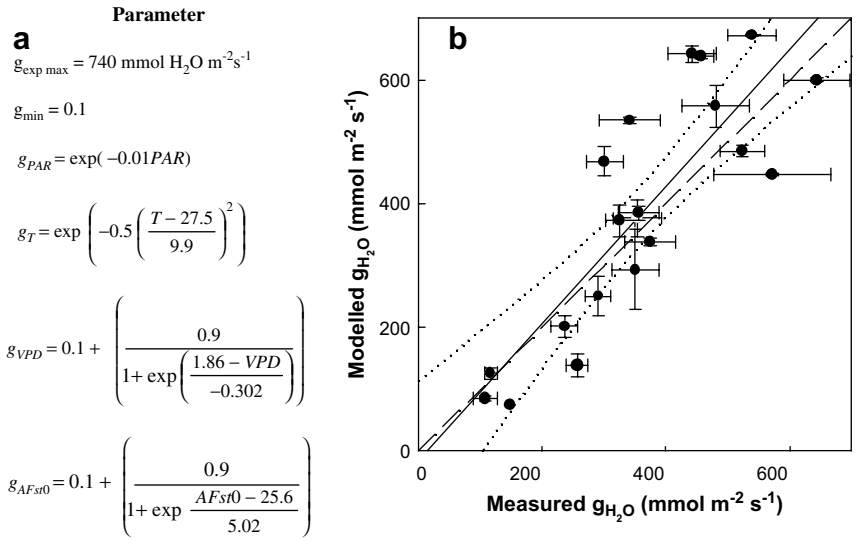

Fig. 3. Algorithms derived from boundary line analysis of key environmental influences on the stomatal conductance (g_{H_2O}) of white clover (a). Regression plot (solid line) of modelled stomatal conductance versus measured stomatal conductance values (g_{H_2O}) using hourly average data (b). Mean shown ± SE, 95% confidence intervals shown for regression (dotted lines). The dashed line shows the $x = y$ reference line.

for the slope and intercept of linear regressions were overlapping). Differences in biomass yield between nutrient treatments were standardized by calculating values relative to their respective NFA counterparts. Variations in the threshold considered resulted in minor influences on the significance of the fitted regressions. Table 5 provides a summary of AOT40–yield relationships. There was no exposure–response relationship evident with respect to the productivity of ryegrass and ryegrass/clover mixtures, but forage yield of clover monoculture was inversely related to AOT40, with the relationship ($n = 12$) increasing in significance as the season progressed ($r^2 = 0.22$ (ns, H1), 0.49 ($p = 0.01$, H2) and 0.59 ($p = 0.004$, H3)). No ozone exposure–response relationships existed for RFV, but relative IVDMD (i.e. IVDMD relative to NFA; a parameter determined only for clover monocultures) appeared to be positively related to increasing ozone (regression analysis revealed $r^2 = 0.42$, $p = 0.02$). A significant relationship was also found between AOT40 and CFV ($r^2 = 0.74$, $p < 0.001$, $n = 12$), though in this case a decreasing exponential relationship appeared the most appropriate fit to the data set given the residuals of the linear regression. Interestingly, analysis of ozone-exposure response relationships revealed a greater decline in clover yield in monocultures versus mixtures (Fig. 4), reflecting a significant ozone × sward composition interaction on biomass yield, statistically significant by H3 ($O_3 \times C$ $p = 0.05$).

Modelled dose–response relationships for clover were first examined using a range of ozone thresholds ($AF_{st}Y$, where Y was tested from 0 to 12 nmol m^{-2} s^{-1}) but the r^2 varied (max. 6%) with changing thresholds. The best predictor of responses was found to be $AF_{st}8$, and Table 5 provides a summary of regression outcomes from analyses employing $AF_{st}8$ to probe impacts on clover yield and quality characteristics. As was found for AOT40, dose–response relationships ($n = 12$) for biomass yield increased in strength as the season progressed ($r^2 = 0.23$ ns, H1), 0.48 ($p = 0.01$, H2) and 0.60 ($p = 0.003$, H3). Moreover, a positive relationship was found between ozone uptake and relative non-digestible fibre content from the IVDMD assay ($r^2 = 0.39$, $p = 0.03$, $n = 12$) and a strong negative relationship existed between ozone uptake and CFV ($r^2 = 0.79$, $p < 0.001$, $n = 12$).

Table 5
Ozone exposure (AOT40) and dose ($AF_{st}8$)–response relationships for clover productivity and quality-related traits

Parameter	AOT40		$AF_{st}8$	
RDW HARVEST 1[a]	$y = -0.003x + 100$	$r^2 = 0.20$, $p = 0.14$	$y = -2.41x + 100$	$r^2 = 0.23$, $p = 0.1$
RDW HARVEST 2[a]	$y = -0.004x + 100$	$r^2 = 0.48$, $p = 0.01$	$y = -4.89x + 100$	$r^2 = 0.48$, $p = 0.01$
RDW HARVEST 3[a]	$y = -0.003x + 100$	$r^2 = 0.58$, $p = 0.004$	$y = -5.95x + 100$	$r^2 = 0.60$, $p = 0.003$
Relative IVDMD[b]	$y = 0.003x + 102$	$r^2 = 0.40$, $p = 0.027$	$y = 7.03x + 104$	$r^2 = 0.39$, $p = 0.03$
CFV[b]	$y = -0.003x + 100$	$r^2 = 0.76$, $p < 0.001$	$y = -7.2x + 101$	$r^2 = 0.79$, $p < 0.001$

Mesocosms were exposed in to four ozone regimes delivering AOT40s of 60 (NFA), 3900 (NFA25), 9450 (NFA40) and 17,160 (NFA55) ppb h in three (replicate) open-top chambers per ozone treatment. RDW, relative biomass dry weight; IVDMD, non-digestible fibres from the in vitro dry matter digestibility assay; CFV, consumable food value.
All regressions used chamber averages ($n = 12$).
[a] Linear regressions combining plants under high and low nutrition fertilization grown in monocultures and mixtures.
[b] Regressions combining high and low fertilized mesocosms.

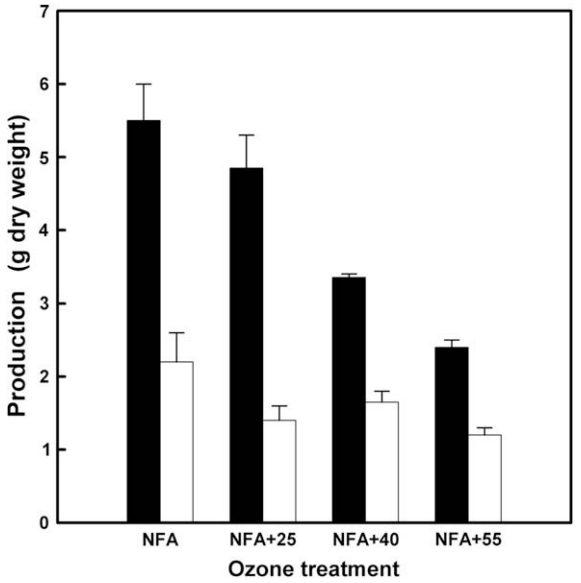

Fig. 4. Interactive effects of ozone and sward composition on clover biomass production – harvest 3 data. Mesoscosms were exposed in three (replicate) open-top chambers per treatment to four ozone regimes delivering AOT40s of 60 (NFA), 3900 (NFA25), 9450 (NFA40) and 17,160 (NFA55) ppb h over the course of the season. Error bars represent the standard error of the mean ($n = 3$).

Subtle differences were evident in AOT40 and $AF_{st}8$ relationships with yield and relative IVDMD, but other approaches suggested the indices performed equally well in terms of regression fits (r^2, p-values and properties of residuals i.e. normality and homogeneity of variance). Arguably the most suitable parameter used to describe treatment impacts on grasslands, CFV (which integrates effects on biomass production and quality parameters) yielded a slightly better relationship with ozone dose ($AF_{st}8$) than exposure (AOT40).

4. Discussion

In the present study, mesocosms containing monocultures or a mixture of perennial ryegrass/'NC-S' white clover were grown at two levels of soil nutrition and exposed in OTCs to four levels of ozone fumigation in order to explore impacts on forage nutritive quality and productivity. The scheduling of the fumigation corresponds with the latter part of the natural growing season in the North of England, and circumvented issues associated with the impacts of low spring temperatures on clover establishment. In agreement with previous grassland studies (Nussbaum et al., 1995; Bassin et al., 2007; Bender et al., 2006), well-fertilized perennial ryegrass proved to be rather unresponsive to ozone. Interestingly, however, ryegrass subject to the 'low nutrient' regime (exhibiting depressed foliar levels of N, K, Zn and increased C/N ratio compared with plants subject to 'high nutrient' treatment) exhibited a significant ozone-induced decline in forage nutritive quality, and this effect was reflected in a significant decline in the most agriculturally relevant parameter CFV, which integrates treatment effects on forage productivity and quality.

In contrast, 'NC-S' white clover, a clone selected for its 'sensitivity' to the pollutant in terms of effects on cumulative dry matter production and visible symptoms (Heagle et al., 1995) and widely adopted within Europe as a biomonitor of ozone impacts (Mills et al., 2006), exhibited a marked ozone-induced depression in productivity and nutritive quality. Detected increases in ADL levels are consistent with the observed accelerated senescence and tissue necrosis (Runeckles and Krupa, 1994) caused by ozone. Whilst ADL content is not included in the calculation of CFV, the percentage of senescent leaves is known to reduce forage nutritive quality through increases in NDF as observed in the present study (Van Soest, 1994). Ozone-induced reductions in nutritive quality caused by accelerated senescence were also reported in a study on *Trifolium striatum* (Sanz et al., 2005). This finding is consistent with some previous reports of the effects of ozone on white clover productivity and forage quality, but a wide range of effects on quality characteristics have been reported, likely due to different methodologies and criteria employed for assessing forage quality (Muntifering et al., 2006). As with several previous observations of the effects of ozone on simple ryegrass/white clover communities (reviewed by Barnes et al., 1999a; Bassin et al., 2007), ozone impacts on total biomass production were not statistically significant, as effects on the clover fraction were compensated by an increase in the yield of ryegrass. In contrast, one of the only long-term free air ozone enrichment studies conducted to date revealed a 23% depression in productivity after five years' exposure to pollution levels equivalent to the NFA + 55 ozone treatment administered in the present study, and a strong decrease in the legume fraction (up to 80% reduction in legume contribution to total biomass) (Volk et al., 2006). Another interesting outcome of the present study was the observation that clover productivity was less affected by ozone in mixed mesocosms than in monocultures (see Fig. 4), suggesting that the presence of the grass in some way reduced ozone uptake by the clover and/or encouraged foliar protection against ozone-induced oxidative stress. Previous studies report variations in environmental conditions dependent on grass/clover balances and canopy structure including shifts in PAR, wind speed and/or ozone concentrations (Lambers et al., 1998; Jäggi et al., 2006; Bassin et al., 2007) consistent with the possibility that the denser canopy of the mixed mesocosms results in reduced O_3 uptake compared with plants grown in monoculture.

Since the vast majority of attention to date has focused on the impacts of ozone pollution on grassland productivity rather than nutritive quality for grazing animals, particular attention was paid to the latter aspect in the present study. A high proportion of clover in the pasture is considered favourable because of its palatability, digestibility and nutritive value for ruminant animals (Van Soest, 1994). Consistent with this view, ryegrass forage contained significantly more NDF and ADF than did clover, and hence RFV were higher for clover than ryegrass. Ozone-induced losses in nutritive quality of clover were in the same range (ca. 15% in NFA + 25) as those reported in another study on the same species employing a free-air fumigation system to expose vegetation to elevated levels of ozone (Muntifering et al., 2006) and in a review of parallel studies with a range

of ozone-sensitive grassland species exposed to ozone in a variety of chamber types (Krupa et al., 2004). There are contradictory reports as to the effects of nutrient status on the impacts of ozone on semi-natural vegetation (Bassin et al., 2007), but in the present study, there was little evidence of significant ozone × nutrient status interactions on grass/clover productivity and forage quality (sensu Sanz et al., 2005). Given the growing interest in the establishment of ozone exposure–response and, latterly, dose–response relationships for grasslands, it is important to recognize the need to consider effects not only on biomass productivity but also nutritive quality, and to integrate both of these effects in future risk assessment approaches (Bender et al., 2006). Given the agricultural importance of CFV, this parameter may be more relevant as a basis for the establishment of exposure–response and/or dose–response relationships than productivity alone (Krupa et al., 2004).

A simplified model to calculate g_{H_2O} (considering the effects of *T*, VPD, PAR and $AF_{st}0$ on g_{max}) was adopted in the present study, as this approach explained 72% of the variation in the measured data set, and regression analysis revealed a low RMSD. Inclusion of other parameters influencing g_{H_2O} resulted in no significant improvement in model behaviour. The derived model showed a tendency to over-estimate measured values, as with many other similar derivations for other species (Danielsson et al., 2003; Emberson et al., 2005). Measured stomatal conductance values (made in this study on the abaxial surface) are within the range of reported maximum values in the literature for white clover, and consistent with Morison's (1998) report that the ratio of abaxial to adaxial stomatal conductance in *Trifolium* spp. ranges from ca. 1.5 to 2 across a range of atmospheric CO_2 concentrations. Based on Morison's (1998) findings there is the possibility that fluxes derived in the present study may slightly underestimate total flux, as a consequence of the non-consideration of adaxial stomatal conductance. However, the g_{max} value derived in the present study (740 mmol H_2O m^{-2} s^{-1}) was in the same range as that derived by Büker (2008) from an extensive measurement campaign conducted on the same clover clone between 1995 and 2002 (resulting in more than 9000 data-points for the derivation of g_{max} for 'NC-S' clover). Our g_{max} was also close to that derived by Mills et al. (2002) from ICP clover clone experiments performed on potted plants over a range of climatic conditions across Northern Europe. Boundary line fits to key drivers of g_{H_2O} were similar to those published previously for clover (Emberson et al., 2000; Mills et al., 2002; Karlsson et al., 2004a), although derived equations (Fig. 2) were experiment-specific. The approach adopted in the present study improved upon the multiplicative g_{H_2O} model derived by Mills et al. (2002); explaining an additional 33% of the variation in the measured data set and reduced the RMSD by 0.64 units when applied to the present data-cloud.

Concerns have been raised about the influence of OTCs on microclimatic conditions and the importance of establishing the way in which such effects modify pollutant uptake by plant canopies has been highlighted (Pleijel et al., 2007). Mean seasonal increases in air temperature of less than 1 °C, 12% reductions in PAR, increases of <5% in RH and reductions in leaf boundary layer resistance were recorded during the U.S. National Crop Loss Assessment Network (NCLAN) open-top chamber programme, but meaningful assessment of the impacts of these microclimatic modifications was confounded by site-to-site variation (Heagle et al., 1988). More recently, Nussbaum and Fuhrer (2000) exploring differences in ozone uptake between OTCs and ambient air plots calculated a mean seasonal ratio of ozone uptake OTC:AA of between 0.89 and 1.04, dependent on species and chamber type/operation. The implication being that OTC studies may significantly underestimate ozone fluxes.

Dose–response relationships employing a cut-off threshold of 8 nmol m^{-2} s^{-1} ($AF_{st}8$) (derived using modelled g_{H_2O} values) were compared with exposure–response relationships based on the accumulated hourly exposure to ozone during daylight hours over a threshold aerial concentration of 40 ppb (AOT40). For calculations of both exposure and uptake, the ozone accumulation period was initiated at the beginning of the fumigation as this method has been shown to provide a closer fit to experimental data for ryegrass–clover mixtures than employment of ozone exposure independently for each 28-day harvest interval (Nussbaum et al., 1995). Indeed, this also proved the case in the present study. AOT40 and $AF_{st}8$ performed equally well for clover data, yielding similar r^2 for exposure– and dose–response effects on productivity, but $AF_{st}8$ slightly out-performed AOT40 in predicting ozone impacts on CFV. This finding is consistent with other recent investigations in which vegetation responses have been better predicted by modelled ozone uptake than by atmospheric exposure (Bermejo et al., 2002; Danielsson et al., 2003; Soja et al., 2004; Pleijel et al., 2004, 2007). In this study both indices performed equally well when locally derived relationships were used. However, dose–response relationships derived from this study require validation over a greater range of environmental conditions to test the robustness of comparison between $AF_{st}Y$ and AOTX indices. Previous analysis of experimental data for wheat and potato has indicated that flux-based indices out-perform AOT40 across experiments (Pleijel et al., 2000, 2007). In contrast, Karlsson et al. (2004b) combined data for studies on several tree species and found AOT40 to perform slightly better than flux. This possibly reflects uncertainties in the parameterization of the underlying model of stomatal conductance (Ashmore, 2005), and the need to use a fixed maximum stomatal conductance across sites (Uddling et al., 2004).

Longer ozone exposure on mesocosms containing upland British clover genotypes, plus field plots subject to the normal agronomic practices in the region, are needed to aid our understanding of ozone effects on grassland productivity and feed value. Future studies should also consider extending the gas exchange measurement campaign across treatments to facilitate the development of multilayer stomatal conductance models in order to improve the predictive capacity of resulting stomatal conductance models, and thus improve dose–response relationships.

5. Conclusions

Ozone risk assessments for highly productive temporary leys and semi-natural grasslands need to give consideration to negative effects of air pollution not only on

productivity but also to forage nutritive quality for ruminant animals. The findings presented suggest that yield-based risk assessments conducted to date may underestimate the impacts of elevated ozone on grassland communities, and consequences for animal production. Ozone exposure studies on field plots subject to the normal agronomic practices in the region would help to better understand the impacts of present and future ozone climates on forage quality and production. The present study highlights the need to establish dose–response relationships for more-complex plant communities in the field, and to scale up from leaf-level stomatal ozone uptake models to canopy-scale algorithms.

Acknowledgements

The authors thank Alan Davison, Colin Gillespie, Alan Craig and Robert Hodgson (Newcastle University – field experimentation) and John Lin (Auburn University – forage quality analysis) for their input. The study was co-funded by the Natural Environment Research Council (GT04/98/304/TS), The Centre for Ecology and Hydrology, Bangor, the European Commission (FP4 BIOSTRESS Project EVK2-CT-1999-00040) and the Alabama Agricultural Experiment Station (Multi-State Project NE-1013).

Appendix. Accumulated ozone stomatal flux (AF_{st})

$$AF_{st} = \sum \left(c_{O_3} g_{H_2O} \; D_{H_2O/O_3} \frac{r_c}{r_b + r_c} \right) 3600^{*} 10^{-6} \qquad (1)$$

where AF_{st} is the accumulated ozone flux (mmol m^{-2}), g_{H_2O} is the stomatal conductance to water vapour calculated according to Eq (1) and D_{H_2O/O_3} represents the difference in diffusivity between H_2O vapour and O_3 in air (0.613; Nobel, 1983). Ozone concentration (c_{O_3}, nmol m^{-3}) was considered as hourly averages and measurements were made at the top of the plant canopy. r_c is the leaf surface resistance accounting for both non-stomatal and stomatal (g_{H_2O}) deposition of ozone and r_b represents the quasi-laminar boundary layer resistance, as described in the Mapping Manual of the LRTAP Convention 2004 (UNECE, 2004).

Consumable food value (CFV)

$$CFV(\%) = 100(RDW^{*}RFV)$$

where RDW is the dry biomass yield relative to the yield of the NFA treatment and RFV represents the relative food value.

Digestible dry matter (DDM)

$$\%DDM = 88.9 - (0.779ADF)$$

where ADF represents the acid detergent fraction (%).

In vitro dry matter digestibility (IVDMD)

NDF analysis was coupled with in vitro fermentation of forage using batch cultures of live ruminal microorganisms (sensu Tilley and Terry, 1963). Non-digestible fibres were determined from the neutral detergent-insoluble residue remaining after 48 h of fermentation.

Relative food value (RFV)

$$RFV = (DDM^{*}DMI)/1.29$$

where DDM and DMI are the percentage of digestible dry matter and the voluntary dry matter intake, respectively, calculated by reference to a digestible DM intake that has been adopted to standardize a forage containing 53% NDF and 41% ADF to a RFV of 100.

Stomatal conductance model

$$g_{mod} = g_{max} MAX\{(g_{PAR} g_T g_{VPD} g_{AF_{st}0}); g_{min}\}$$

where g_{mod} is the modelled g_{H_2O} value, $g_{max} = 740$ mmol $H_2O\ m^{-2}\ s^{-1}$ is the maximum g_{H_2O} value under the experimental conditions prevailing, g_{min} the minimum g_{H_2O} registered under the experimental conditions prevailing and g_{PAR}, g_T, g_{VPD} and $g_{AF_{st}0}$ represents the functions derived from boundary line analyses relating to the influence of PAR, T, VPD and $AF_{st}0$, respectively, on stomatal conductance (relative to g_{max}).

Voluntary dry matter intake (DMI)

$$DMI = 120/NDF$$

where NDF represents the neutral detergent fraction (%).

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5. MODELIZACIÓN DE LA DINÁMICA DE PASTOS ANUALES: APLICACIÓN AL CÁLCULO DEL DEPÓSITO ESTOMÁTICO DE OZONO

González Fernández I, Bermejo, V, Alonso R, Elvira S, Sanz J, Gimeno B S. (2009). Modelling dehesa annual pasture dynamics: application to stomatal ozone deposition. Global Change Biology, submitted.

Resumen

La modelización del depósito estomático del ozono (O_3) para el análisis de riesgos de efectos del O_3 en la vegetación se encuentra poco desarrollada para la vegetación herbácea, particularmente para los pastos mediterráneos anuales. El área mediterránea se caracteriza por una elevada variabilidad climática interanual que influye sobre la dinámica de la vegetación. Esta variabilidad dificulta el desarrollo de modelos que caractericen de manera adecuada el intercambio gaseoso y la absorción de los contaminantes necesarios para realizar un análisis de riesgos. Este artículo presenta un nuevo modelo para estimar la conductancia estomática (g_s) del *Trifolium subterraneum*, una especie característica de los pastizales que crecen en las dehesas. El modelo MEDPAS (*MEDiterranean PAStures*) acopla tres módulos que estiman el contenido de agua en el suelo, el crecimiento de la vegetación y la g_s. El módulo de g_s es una versión reparametrizada del modelo de g_s incluido en la función de depósito de ozono del modelo fotoquímico del EMEP (DO_3SE).

El modelo MEDPAS se aplicó a las condiciones de dos años, 2005 y 2007, que representan las condiciones típicas de una primavera seca y húmeda respectivamente. Ambos años presentaron además una exposición al O_3 también diferente. El modelo MEDPAS reprodujo de forma realista las variaciones estacionales e interanuales de g_s observadas en el campo, mejorando los resultados obtenidos cuando se utilizaban parametrizaciones del módulo de g_s específicas para cada año. Estas parametrizaciones específicas para cada año eran necesarias para poder representar la variabilidad interanual observada en la g_s, dificultando su aplicación para el cálculo de los flujos estomáticos de O_3 en los pastos de dehesas.

El contenido hídrico del suelo fue identificado como uno de los factores más importantes que explican las diferencias observadas en la dinámica de la vegetación

entre los dos años. Aunque la exposición al ozono fue más elevada en el 2005, las condiciones meteorológicas favorecieron valores de g_s hasta 2,1 veces más altos y un periodo de crecimiento 56 días más largo en el 2007, lo que se tradujo en que los flujos de O_3 absorbidos por *T. subterraneum* fueron más elevados ese último año.

Se detectó una elevada variabilidad inter-específica en parámetros clave para el cálculo de g_s, por lo que los flujos de O_3 calculados para *T. subterraneum* deben considerarse como un valor promedio. Además, los resultados indicaron que la influencia del clima en el crecimiento y la conductancia estomática del pasto debe tenerse en cuenta con el fin de poder modelizar de forma más precisa los flujos estomáticos de O_3 para los escenarios climáticos presentes y futuros en el área mediterránea.

MODELLING DEHESA ANNUAL PASTURE DYNAMICS: APPLICATION TO STOMATAL OZONE DEPOSITION

Running title: Ozone stomatal uptake in dehesa pastures

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Keywords: climatic inter-annual variability, drought, soil water content, pasture growth, phenology, stomatal conductance, stomatal ozone flux, risk assessment

Abstract

Modelling ozone (O_3) deposition for impact risk assessment is still poorly developed for herbaceous vegetation, particularly for Mediterranean annual pastures. The Mediterranean area is characterized by a high inter-annual climatic variability that influences vegetation dynamics. This variability makes difficult to develop models characterizing gas exchange behaviour and air pollutants absorption suitable for risk assessment. This paper presents a new model to estimate stomatal conductance (g_s) of *Trifolium subterraneum*, a characteristic species of dehesa pastures. The MEDPAS (MEDiterranean PAStures) model couples 3 modules estimating soil water content (SWC), vegetation growth and g_s. The g_s module is a reparameterized version of the g_s model included in the European EMEP ozone deposition model (DO_3SE). The MEDPAS model was applied to two contrasting years, 2005 and 2007, representing typical dry and humid springs respectively and with different O_3 exposures. The MEDPAS model reproduced realistically the g_s seasonal and inter-annual variations observed in the field, outperforming year-specific parameterizations of the g_s module. Reparameterizations of year-specific models were needed to meet the inter-annual variability observed in g_s, making them unsuitable for O_3 flux modelling for dehesa pastures. SWC was identified as the major driver of differences across years. Despite higher O_3 exposure in 2005, meteorological conditions favoured 2.1 times higher g_s and

56 days longer growing season in 2007 compared to 2005, resulting in higher ozone fluxes absorbed by *T. subterraneum*. High inter-family variability was found in key parameters of the g_s module, therefore calculated fluxes for *T. subterraneum* represent an average value. Weather influence on pasture growth and g_s dynamics need to be considered for more accurate O_3 flux modelling for present and future climate scenarios in the Mediterranean area.

1. Introduction

Over recent decades, air pollution abatement policies have resulted in a reduction in peak ozone (O_3) concentrations across Western Europe (Jonson *et al.*, 2006). However, background levels of O_3 pollution have increased globally and could continue rising in the Northern hemisphere if current emission trends persist (Dentener *et al.*, 2006). Ozone is still a pollutant of major concern due to its wide distribution and its current ambient levels high enough to cause effects on growth, reproductive development or competitive ability on a number of plant species (Davison & Barnes, 1998; Black *et al.*, 2000; Bermejo *et al.*, 2003; Fuhrer & Booker, 2003; Gimeno *et al.*, 2004a,b). Moreover, a range of O_3-induced changes in composition, diversity and productivity of natural and semi-natural plant communities have been identified (Davison & Barnes, 1998; Krupa *et al.*, 2004; Volk *et al.*, 2006; Bassin *et al.*, 2007).

In Europe, O_3 critical levels have been proposed for the protection of crops, forests and semi-natural vegetation under the framework of the Convention on Long-Range Transboundary Air Pollution (CLRTAP; UNECE, 2007). The semi-natural vegetation term includes an enormous amount of very diverse plant community types with complex interactions among species and environmental factors, increasing the difficulties for defining O_3 critical levels (Bassin *et al.*, 2007). Ozone critical levels for risk assessment and target values established for the protection of vegetation in the European legislation (Directive 2008/50/EC) are based on the cumulative O_3 atmospheric concentrations above a threshold of 40 ppb during daylight hours (AOT40 index). However, there is substantial experimental evidence that responses of plants are more closely related to the O_3 dose absorbed through the stomata than to O_3 exposure

(Ashmore *et al.*, 2007). As a consequence, considerable attention has focused on the development of flux-based critical levels and the models needed to estimate stomatal O_3 fluxes. Particular efforts have been made on the parameterization of the stomatal component of the DO_3SE model (Emberson *et al.*, 2000), incorporated into the EMEP (European Monitoring and Evaluation Programme) model, used to predict O_3 deposition across Europe within the CLRTAP (Ashmore *et al.*, 2007).

Dehesas are savannah-like cleared woodlands representing one of the most characteristic landscapes of South-Western Spain and Portugal. Their productivity covers agricultural, timber and extensive livestock exploitation. These pastures present a remarkably high plant species richness and they are included among the protected areas in the Nature 2000 Network for biodiversity conservation (European Directive 92/43/EEC). Dehesa pastures are dominated by annual species, whose floristic composition and dynamics varies largely in space and time depending on environmental factors (topography, meteorological and edaphic conditions), grazing management and tree coverage (Ortega *et al.*, 1997; Peco *et al.*, 1998, 2006). Among annual Mediterranean species, legumes are important components in the nitrogen cycling and represent a valuable nutritive source for livestock in dehesa pastures.

Although the Mediterranean region is frequently exposed to ambient O_3 levels reported as phytotoxic for annual species (Bermejo *et al.*, 2003; Gimeno *et al.*, 2004a), the number of studies focusing on O_3 effects on dehesa pastures is still scarce. Less than 20% of the species characteristic of these communities have been screened for O_3 sensitivity in terms of visible injury or biomass loss, showing a great variability (Bermejo *et al.*, 2003; Gimeno *et al.*, 2004a), and just a few have been used to study O_3 impacts on other traits such as reproduction ability or forage quality (Gimeno *et al.*, 2004b; Sanz *et al.*, 2005, 2007). Particularly little information is available about ecophysiology and gas exchange behaviour of dehesa pastures for O_3 risk assessment based on stomatal fluxes. Previous field measurements of gas exchange rates have revealed a great inter-specific variation in key parameters of the DO_3SE stomatal gas exchange model in a dehesa grassland in central Spain (Alonso *et al.*, 2007). Furthermore, plant dynamics vary greatly from year to year in the Mediterranean area, influenced by the characteristic high inter-annual variability in meteorological

conditions (Peñuelas *et al.*, 2004; Montaldo *et al.*, 2008; Peco *et al.*, 2009). Modelling approaches to calculate O_3 fluxes for risk assessment need to cover the variability associated with annual pasture dynamics.

In the present paper, field-based gas exchange measurements in naturally growing dehesa pastures are analyzed with the following objectives: (1) characterize the factors controlling stomatal conductance, plant growth, leaf area index (LAI) dynamics and length of the growing season of dehesa pastures; (2) explore the variability in the maximum stomatal conductance value at leaf level in annual Mediterranean species, a key parameter of the DO_3SE model; and (3) derive a model to describe annual pastures dynamics using a simple, low input approach by means of coupling the DO_3SE with two additional modules describing soil water balance and vegetation growth and to use the derived model, hereafter referred as MEDPAS model, for calculating O_3 stomatal fluxes for risk assessment in annual dehesa pastures.

2. Materials and methods

2.1 Study site and environmental conditions

The study site was located in a dehesa ecosystem in Miraflores de la Sierra (40º 48.4' N, 3º 48.4' W; 1059 m.a.s.l), 50 Km northeast of Madrid city (central Spain). This dehesa is a *Quercus ilex* subsp. *ballota* (Desf.) open woodland devoted to extensive cattle feeding. The climate is continental Mediterranean, characterized by relatively cold and humid winters followed by warm dry summers. The characteristic high inter-annual weather variability makes unpredictable the start and length of the summer drought. Mean annual rainfall (1961-1990) was 720 mm with a very irregular distribution throughout the year.

Hourly O_3 concentration and meteorological variables were collected from the closest air quality monitoring station of the Air Quality Control Network operated by the regional government of the Comunidad Autónoma de Madrid, located at Colmenar Viejo. The 2004/05 growing season was dryer, warmer and sunnier and with higher O_3 concentrations than the 2006/07 season (Table 1).

Year	Season	PAR ($\mu mol\ m^{-2}\ s^{-1}$)	T (ºC)	VPD (kPa)	P ($l\ m^{-2}$)	AOT40 (ppb.h)
2004/2005	Autumn	553 ± 12	10.1 ± 0.5	0.46 ± 0.05	164	1321
	Winter	652 ± 12	5.3 ± 0.4	0.44 ± 0.02	46.5	2800
	Spring	901 ± 15	16.2 ± 0.6	1.1 ± 0.07	80	14791
	Summer	957 ± 14	23.2 ± 0.4	2.1 ± 0.07	17.9	18000
2006/2007	Autumn	524 ± 13	11.0 ± 0.5	0.4 ± 0.04	353	505
	Winter	583 ± 13	6.5 ± 0.3	0.32 ± 0.02	22	732
	Spring	816 ± 15	13 ± 0.5	0.7 ± 0.04	246	5617
	Summer	950 ± 15	21.5 ± 0.3	1.8 ± 0.06	14	4298

Table 1. Meteorological conditions during 2004/2005 and 2006/2007 growing seasons. Values shown are daylight (solar radiation > 100 W m^{-2}) averages ± SE of photosynthetic active radiation (PAR), daily averages ± SE of temperature (T) and vapour pressure deficit (VPD). Precipitation (P) represents the accumulated rainfall during the season. Accumulated ozone concentrations above a threshold of 40 ppb (AOT40) were calculated during daylight hours for 3 months.

2.2 Gas exchange measurements

Stomatal conductance to water vapour at leaf level (g_s) was measured using a LICOR-6400 infrared gas analysis system (LiCor Inc., Lincoln, NE, USA). Fully expanded leaves were selected for gas exchange measurements performed under prevailing environmental conditions of PAR, relative humidity (RH) and temperature (T), which were recorded simultaneously with g_s. Field work started before flowering onset and continued until leaf senescence, yielding a database of 326 and 435 individual leaf measurements in 2005 and 2007 respectively. The gas exchange field assay in 2005 has been described previously in Alonso *et al.* (2007). It was focused on four of the most abundant species, 2 grasses (*Bromus hordeaceus, B. sterilis*) and 2 legumes (*Trifolium striatum, T. subterraneum*).

In 2007, the field work spanned 10 weeks, from April 20th to June 27th. Gas exchange measurements were performed under sunny conditions during the central hours of the day. Cuvette temperatures were in the range 17 – 35ºC, while VPD and PAR ranged 0.5 – 4.4 kPa and 100 – 2300 $\mu mol\ m^{-2}\ s^{-1}$ respectively. Between 1 and 13 plants per species were measured each day, although not all the species were measured every sampling day. In total, g_s was determined for 6 different families (*Cistaceae*, *Compositae*,

Geraniaceae, *Gramineae*, *Leguminosae* and *Plantaginaceae*). Only two species, *Bromus hordeaceus* and *Trifolium subterraneum*, were extensively measured both years. Stomatal conductance values are expressed on a projected leaf area (PLA) basis. Leaf area was determined using Win FOLIA software (Régent Instruments Inc., Canada).

2.3 Soil moisture and plant biomass

Soil water content (SWC) was measured with quasi-time-domain reflectometry technique (TDR) (TRIMEGM, IMKO GmbH, Germany) at the same time as gas exchange determinations. Four to seven measurements at 0-15 cm depth were performed at five independent plots representing the study area.

Plant biomass was determined only in 2007 throughout the spring growing season. Between three and five disks of 0.05 m^2 were placed randomly over the pasture each sampling day and all the individuals within the area were collected. Plant material was dried in the oven at 65ºC until constant weight, classified per family and weighted to determine dry biomass weight per family.

2.4 Model description

A new MEDPAS (MEDiterranean PAStures) model has been developed to describe growth, gas exchange behaviour and air pollutants absorption on Mediterranean annual pastures. The MEDPAS model couples three different modules estimating soil water availability, growth and g_s respectively in order to calculate O_3 deposition fluxes (Figure 1). The soil water availability module estimates soil water content (SWC) on a daily basis by means of a soil water balance depending on meteorological variables and soil properties. Calculated available soil moisture is used as an input for the growth module, which predicts vegetation growth on a daily scale (B, biomass). Daily B values are used to compute daily leaf area index (LAI) and canopy height (h), in turn, modulating the evapotranspiration component (ET_c) of the SWC module. Also, the growth module served to establish the length of the growing season of these communities. SWC and biomass are computed on a daily basis throughout the hydrological year from October 1st to September 30th. Calculated SWC together with meteorological input data are used to model hourly g_s values using the multiplicative approach described by Jarvis (1976) and modified by Emberson *et al.* (2000) that is included in the EMEP DO_3SE deposition module. Finally stomatal O_3 fluxes are

calculated using the DO_3SE deposition module for the period defined by the growth module.

Trifolium subterraneum was used as a representative dehesa annual species for estimating stomatal O_3 fluxes. This species was selected due to its reported O_3 sensitivity in terms of aerial and subterranean biomass production, reproductive ability and forage quality (Gimeno *et al.*, 2004a,b; Sanz *et al.*, 2005) and because legumes represented up to 35% of the total dry weight biomass of the community in the 2007 field study.

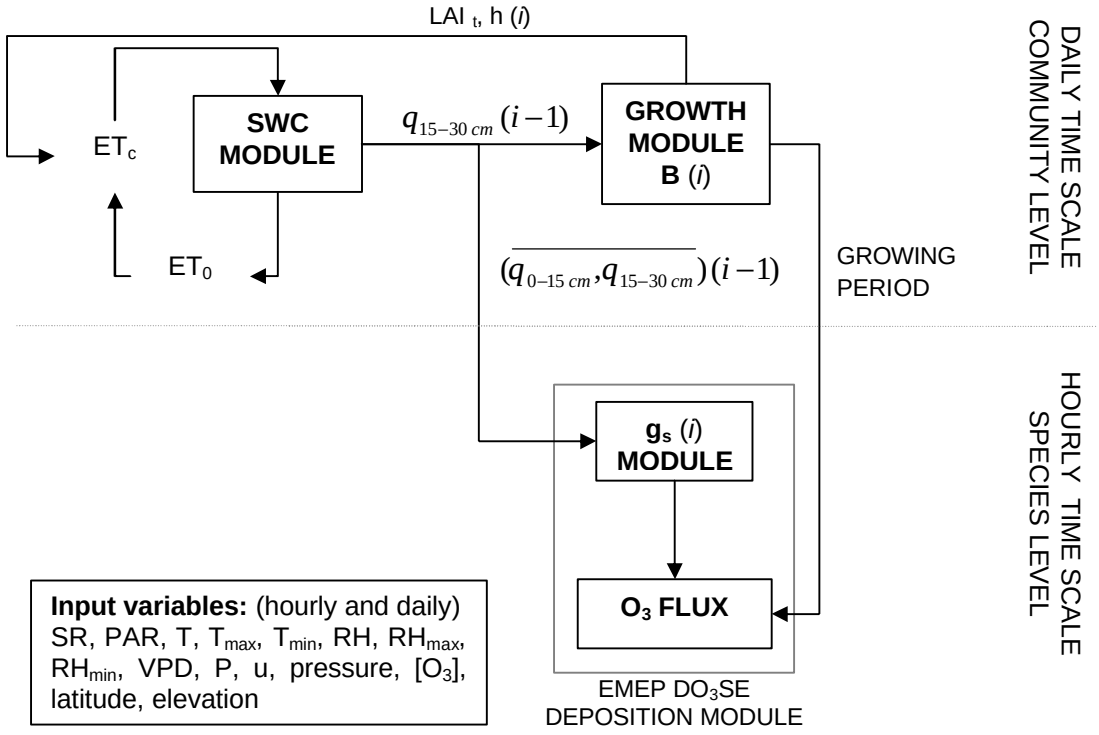

Figure 1. MEDPAS model diagram. Community level soil water content (SWC) and pasture growth modules were used at a daily time scale, and the species-specific stomatal conductance (g_s) module at an hourly time scale. Main input variables that coupled the modules are specified in the arrows connecting the modules. Note that the growth and the g_s modules compute values for day *"i"* using the SWC at the end of day *"i - 1"*. ET_0, reference evapotranspiration; ET_c, actual evapotranspiration; $?_{15-30\,cm}$, soil water content in the 15 – 30 cm depth soil layer; LAI_g, green leaf area index; h, canopy height; $(\overline{q_{0-15\,cm}\ q_{15-30\,cm}})$ average soil water content of 0 – 15 cm and 15 – 30 cm depth soil layers.

2.4.1 Soil water content module

The SWC module calculates daily SWC (?) at two different layers 0-15 and 15-30 cm deep, $?_{0\text{-}15\ cm}$ and $?_{15\text{-}30\ cm}$ respectively. The SWC module consists of a two-layered

bucket model following Nouvellon *et al.* (2000). The two layers were selected to account for the soil depth affected by both evaporation and plant transpiration processes (evapotranspiration, ET_c), set down to 15 cm as recommended in Allen *et al.* (1998), and the soil layer where only transpiration losses (T_c) occur, which is equivalent to the lower end of the rooting zone. The rooting depth was set at 30 cm for annual communities (Moreno *et al.*, 2005).

After a rain event, the water infiltrating into the soil is distributed down the soil profile filling the two layers successively from top to bottom after ? in the upper layer reaches the field capacity. The soil water balance follows the scheme described by Allen *et al.* (1998):

$$\left.\begin{aligned} q_i(0-15cm) &= q_{i-1}(0-15cm) + (P_i - Int_i - RO_i) + I_i + CR_i - ET_{ci} - DP_i(15cm) \\ q_i(15-30cm) &= q_{i-1}(15-30cm) + DP_i(15cm) + CR_i - T_{ci} - DP_i(30cm) \end{aligned}\right\} \quad (1)$$

where $?_i$ and $?_{i-1}$ are the soil water content at the end of days *i* and *i*-1 respectively in each soil layer. Soil water balance depends on the amount of water gained or lost by the soil layer at day *i* due to precipitation (P_i), canopy interception loss (Int_i), run-off (RO_i), net irrigation (I_i), capillary rise (CR_i) from the water table, evapotranspiration ($ET_{c\,i}$), plant transpiration ($T_{c\,i}$) and deep percolation (DP_i). All the units are given in mm.

From the water reaching the surface during a rain event, a certain amount is hold in the canopy due to rain interception. An interception loss (Int_i) by the pasture canopy was calculated from LAI values following (White *et al.*, 2000):

$$Int_i = 0.0225 \cdot (2 \cdot LAI_i) \cdot P_i \quad (2)$$

where LAI_i values are obtained from the growth module and P_i from the weather monitoring station.

The rain infiltrating into the first soil layer ($P_i - Int_i$) is added to the soil water storage from the previous day ($?_{i-1}$). In the first layer (0-15 cm depth), the soil water is mainly lost as ET_{ci} and DP_i(15cm). ET_c is calculated as the sum of direct soil evaporation and plant transpiration following the standard FAO Penman-Monteith methodology described in Allen *et al.* (1998). When the infiltration input fills the water holding capacity, the water is lost from the surface by DP_i (15 cm) in benefit of the 15 – 30 cm soil layer. DP_i (15 cm) is calculated as the excess of infiltrated water when the upper

soil layer is already at field capacity. Main losses in the second layer (15-30 cm) are T_{ci} and DP_i (30 cm). Plants extract water from both soil layers considered. The partitioning of plant transpiration between the two layers was based on the work by Allen *et al.* (2005a) for shallow rooted plants (less than 50 cm rooting depth), and it was proportional to the amount of available water in each layer.

A number of simplifications were made from the water balance in Equation 1. I_i was disregarded since natural pastures are not irrigated, and CR_i and RO_i were also ignored. The water table was considered to be deep enough (more than 1 m) for not contributing through CR_i to the water balance of the upper soil layers (Allen *et al.*, 1998). RO_i in flat areas like the dehesa of this study is considered small in case of light precipitation events. When rains are susceptible of causing runoff, precipitation is sufficient to replenish the water storage of the upper soil layer up to field capacity (Allen *et al.*, 1998) resulting in no net influence on the soil water balance.

Daily SWC values were computed from October 1st, start of the hydrological year, until September 30th of the following year. Initial ? was set to represent a dry soil. Further details of the equations and constant values used in the SWC module are provided in Appendix 1.

Daily SWC values at 15–30 cm depth ($?_{15\text{-}30\ cm}$) were used as input for the growth module, while daily averages of SWC in both layers ($\overline{q_{0-15cm}, q_{15-30cm}}$) were used to calculate the stomatal conductance of *T. subterraneum* using the g_s module (Figure 1).

2.4.2 Pasture growth module

The pasture growth module is a modified version of the vegetation dynamic model described by Montaldo *et al.* (2005, 2008). The growth module computes daily changes of B (g dry matter (DM) m^{-2}) based on rates of carbon gain (photosynthesis) and carbon loss (respiration and senescence) according to the prevailing hydro-meteorological conditions (Equation 3). Two biomass components were simulated: green biomass (B_g) and standing death aerial biomass (B_d). The sum of these two components constitutes the total biomass production (B_t):

$$\left.\begin{aligned} B_g &= a_a \cdot P_g - R_g - S_g \\ B_d &= S_g - L_a \end{aligned}\right\} \qquad (3)$$

where P_g is the rate of carbon gain, a_a is the partitioning coefficient to the leaves, R_g is the respiration rate of leaves, S_g is the senescence rate of leaves, and L_a is the litter fall rate.

B_g was related to LAI (m^2 m^{-2}) and h (m) through relationships taken from Montaldo *et al.* (2008) and Arora & Boer (2005) respectively (see also Appendix 1):

$$LAI_g = c_g \cdot B_g \qquad (4)$$

$$h = c_h \cdot C_L^{0.5} \qquad (5)$$

where LAI_g is the green leaf area index; c_g is the specific leaf area of the green biomass (m^2 g^{-1} DM) taken from Nouvellon *et al.* (2000); c_h is the conversion factor to calculate pasture height (m Kg^{-1} C); C_L is the amount of green biomass (Kg C m^{-2}) calculated by multiplying g DM by a factor of $0.46 \cdot 10^{-3}$ Kg C g^{-1} DM (Larcher, 2003). Further details of the equations and constant values used in the growth model are provided in Appendix 1. Some of the parameters used in this model were selected to meet field observations of biomass production. Stress functions used to estimate P_g (Montaldo *et al.*, 2005, 2008) depending on T (f_2(T)) and VPD (f_3(VPD)) were adjusted to field observations of assimilation at the leaf level, while the parameterization of the soil moisture stress function (f_1(?)) was adopted from the SWC module.

The Montaldo *et al.* (2005, 2008) growth model was modified to specifically describe the phenology of annual species in dehesa pastures, including seed emergence. Two algorithms were added to estimate the start and end of the growing season depending on environmental conditions. To determine the start of the growing period, a sub-model presented by Arora & Boer (2005) was used, where leafing is established when the average carbon gain of a given LAI is positive over seven days:

$$\frac{\sum_{i=n}^{n+6}\left[P_g(LAI_0)\cdot a_a - R_g\right]}{7} \begin{cases} > 0 & Start\ \ leafing \\ \leq 0 & Seed\ \ dormancy \end{cases} \qquad (6).$$

Initial LAI (LAI_0) is calculated as a function of available non-structural carbohydrate reserves, which in annual species are contained inside the seeds. Carbohydrate reserves for initial growth are estimated from the soil seed pool, assuming that the proportion of

species in the seed bank is the same as the proportion of adult plants in the field. LAI_0 is calculated based on the 20% of the leaf biomass (B_{g0}) that the reserves (seeds) could produce according to the root:shoot ratio (Arora & Boer, 2005):

$$\left.\begin{aligned} LAI_0 &= 0.2 \cdot c_g \cdot B_{g0} \\ B_{g0} &= 0.75 \cdot B_{seeds} \end{aligned}\right\} \qquad (7).$$

The mean number of seeds ready to germinate in the soil of central Iberian dehesas has been estimated in 10^5 seeds m^{-2} (Ortega *et al.*, 1997). Seed weights (SW_f) published by Sánchez *et al.* (2002) of the most representative species per family found in the field were used to calculate the seed biomass:

$$B_{seeds}\ (g\ m^{-2}) = 10^5 \cdot \sum (SW_f \cdot abundance_f) \qquad (8)$$

where "*abundance*" is the proportion of biomass of a specific family (*f*) found in the biomass samples collected in the field in 2007. The partitioning of the seed biomass between roots and leaves was equivalent to 0.32, the average root:shoot ratio found in common annual species of dehesa ecosystems (Gimeno *et al.*, 2004a).

For establishing the end of the growing season, a second algorithm was added simulating plant flowering and senescence. Flowering start is calculated based on a double threshold fulfilment of temperature and photoperiod according to Iannucci *et al.* (2008). The threshold values used in this study, based on field observations, were 1880ºC day and 13.5 h of thermal time and photoperiod respectively. Thermal time values were accumulated since the start of the hydrological year (October 1st). A negative leaf carbon balance after flowering during 15 consecutive days triggered the start of the senescence process. This specific lag was selected according to Montaldo *et al.* (2008):

$$\left.\begin{aligned} &\left.\begin{aligned} &\sum Thermal\ \ time > 1880\ ^{o}C\ day \\ &Photoperio\ d \quad > 13.5\ h \end{aligned}\right\}\ Flowering \\ &\sum_{i=n}^{n+14} \left[P_g(LAI_g) \cdot a_a - R_g\right] \quad < 0 \end{aligned}\right\}\ Senescence\ \ start \qquad (9).$$

The senescence process was reflected by an increase in the death rate of green leaves (d_a) up to the value reported by Nouvellon *et al.* (2000) for old water stressed shoots. The litter production (k_a) rate was also increased according to the value used by Montaldo *et al.* (2008).

Two outputs were used to couple the growth module with the SWC and g_s modules (Figure 1): (1) daily values of LAI_g and h were used for calculating the evapotranspiration component (ET_c) of the SWC module; (2) the length of the growing period, from seed emergence to plant senescence, was introduced in the DO_3SE deposition module to adjust the period for accumulating O_3 stomatal flux to the growing season length.

2.4.3 Stomatal conductance module

Stomatal conductance was estimated using the multiplicative approach proposed by Jarvis (1976) and modified by Emberson *et al.* (2000) following the EMEP DO_3SE deposition model:

$$g_s = g_{\max} \cdot f_{light} \cdot f_{phen} \cdot \max\{f_{\min}; (f_T \cdot f_{VPD} \cdot f_{SWC})\} \quad (10)$$

where g_{max} is a constant representing the species-specific maximum stomatal conductance (mol H_2O m^{-2} s^{-1}). The remaining relative functions, ranging from 0 to 1, describe the factors modifying g_{max}: PAR (f_{light}), plant phenology (f_{phen}), temperature (f_T), VPD (f_{VPD}) and SWC (f_{SWC}). f_{min} is the minimum g_s that occurs during daylight hours expressed as relative to the maximum.

g_{max} was calculated as the average of selected values above the 90th percentile of the entire dataset using a minimum n = 3. The coefficient of variation of the mean was kept below 20%, to prevent the inclusion of outliers. f_{light}, f_{phen}, f_T, f_{VPD} and f_{SWC} were defined using the boundary line technique.

Three different parameterizations were tested to model g_s: (1) the parameterization already published for *T. subterraneum* (Alonso *et al.*, 2007) based only in 2005 measurements, referred hereafter as the '2005 model'; (2) a parameterization following the same criteria and methods but based only in 2007 measurements named '2007 model'; and (3) the MEDPAS model. The MEDPAS model includes a g_s module fitting the 2005 and 2007 datasets together. A new f_{phen} *(on/off)* was adopted which weighted the fluxes with a value of one during the pasture growing season or zero otherwise. The start and end of the growing season were obtained from the growth module as described in the previous section. Also a new f_{SWC} was included to describe the effects of soil moisture availability on g_s.

2.5 Stomatal O_3 flux calculation

Accumulated stomatal O_3 fluxes were calculated following the Mapping Manual (UNECE, 2007):

$$AF_{st} = \sum (c_{O_3} \cdot g_s \cdot D_{H_2O/O_3} \cdot \frac{r_c}{r_b + r_c}) \cdot 3600 \cdot 10^{-6}$$

where, AF_{st} is the accumulated O_3 flux (mmol m^{-2}); c_{O3} is the hourly O_3 concentrations (nmol m^{-3}); g_s is the stomatal conductance (m s^{-1}) to water vapour; $D_{H2O/O3}$ represents the difference in diffusivity between H_2O vapour and O_3 in air (0.663; Massman, 1998); r_c (s m^{-1}) is the leaf surface resistance accounting for both non-stomatal and stomatal deposition of O_3; and r_b (s m^{-1}) represents the quasi-laminar boundary layer resistance. Hourly O_3 concentrations measured at 2 m height were corrected to yield concentrations at canopy height following the methodology described in UNECE (2007). For comparison with previous approaches, AF_{st} values for *T. subterraneum* were derived using the three different g_s parameterizations described above.

2.6 Statistical analysis

Soil water content, B_t and g_s modelled values in 2005 and 2007 were compared with field measurements (only 2007 field data available for B_t). Simple regression analyses were used to quantify the relationship between measured and modelled data. The goodness of fit for each model was tested with the R^2 coefficient and the root mean squared error (RMSE). Maximum g_s values selected for the derivation of the g_{max} per family and year were subjected to one-way ANOVA and Tukey HSD post-hoc analysis in order to explore the variability found in the field. Stomatal conductance values were transformed using the Box-Cox transformation when normality and homocedasticity were not met. If transformed variables did not met ANOVA assumptions, then Mann-Whitney U-test was used instead. Differences in the weather conditions (T, PAR and VPD) at the time of the g_{max} measurements between years were compared using MANOVA. Alpha was set at 0.05 for all comparisons. Regression analyses were used to fit the boundary lines when reparameterizations of the existing g_s multiplicative model were needed to fit the data presented in this study. All statistical analyses were performed using Statistica 6.0 (StatSoft Inc., Tulsa, USA).

3. Results

3.1 Inter-specific and inter-annual variability of g_s values

Inter-family and inter-specific variability of g_s was analyzed using the g_{max} as a key parameter of the g_s multiplicative model. Maximum g_s values showed a great inter-family variability ranging from 0.745 to 1.77 mol H_2O m^{-2} s^{-1} (Figure 2a). Significant differences were detected ($p<0.001$, df = 38) with *Geraniaceae* showing the highest g_{max} and *Cistaceae* and *Gramineae* the lowest. Differences on g_{max} among species of the same genus were explored comparing three *Trifolium* species: *T. glomeratum*, *T. striatum* and *T. subterraneum*, measured in 2007. A significant variation ($p<0.001$, df = 11) of nearly 5-fold was detected between the highest g_{max} (*T. subterraneum*, 1.18 mol m^{-2} s^{-1}) and the lowest value (*T. glomeratum*, 0.25 mol m^{-2} s^{-1}) (data not shown). *Trifolium subterraneum*, selected as a representative species to calculate stomatal O_3 deposition, showed a g_{max} value close to the mean g_{max} across plant families, 1.19 mol m^{-2} s^{-1} (Figure 2a).

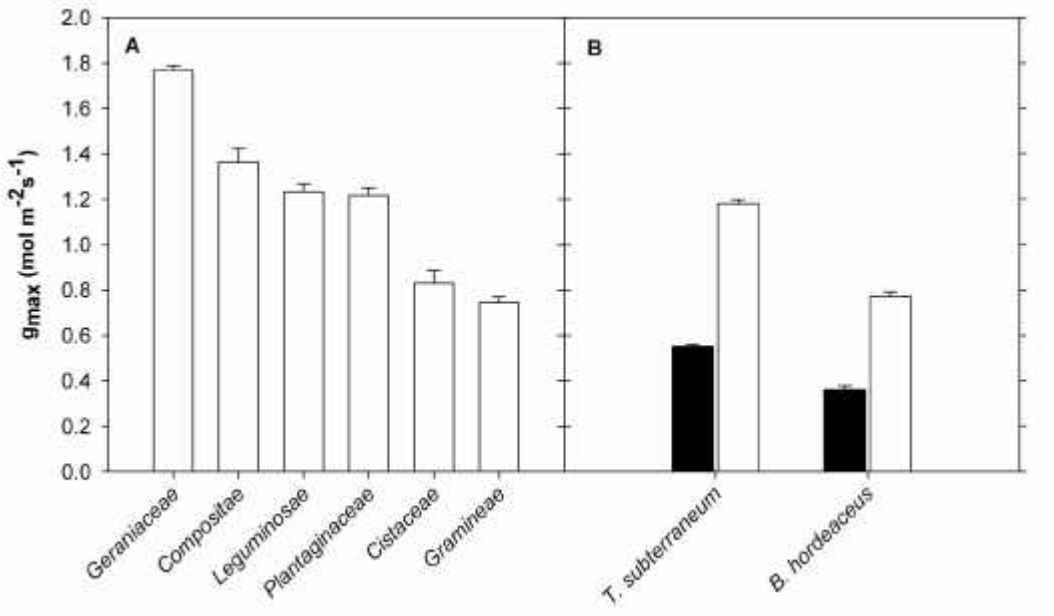

Figure 2. (A) Inter-families variability found on maximum stomatal conductance (mol H_2O m^{-2} s^{-1}) measured in a naturally growing dehesa pasture in central Spain during the spring of 2007. (B) Inter-annual variability found comparing maximum stomatal conductance (mol H_2O m^{-2} s^{-1}) from two species recorded in a naturally growing dehesa pasture during the spring growing season of 2004/05 (black bars) and 2006/07 (white bars): *Bromus hordeaceus* and *Trifolium subterraneum*. Bars represent the mean ± SE.

The inter-annual variability of g_{max} was studied comparing 2005 and 2007 values recorded in the field for *Trifolium subterraneum* and *Bromus hordeaceus*, representative species of legumes and grasses respectively (Figure 2b). Both species showed significantly higher g_{max} values in 2007 compared to 2005 ($p<0.001$, df = 11 for *T. subterraneum*; $p< 0.005$, df = 8 for *B. hordeaceus*). However, no differences were found in predominant microclimatic conditions (T, PAR) at the time of *T. subterraneum* g_{max} measurements. Although significant differences were found in VPD ($p<0.01$, df = 11), values were not limiting g_s in any year. SWC was higher in 2007 than in 2005, differences that were not statistically tested due to the lack of some SWC measurements coupled with g_s data included for g_{max} calculation. Soil water content at 0-15 cm deep averaged 0.06 and 0.18 m^3 m^{-3} at the time of g_{max} measurement in 2005 and 2007 respectively. The lower *Bromus hordeaceus* g_{max} value recorded in 2005 could be related to the lower T ($p< 0.01$, df = 8) recorded at the time of g_{max} (averaging 16ºC) compared to 2007 (averaging 22ºC) and lower soil moisture, averaging 0.05 and 0.19 m^3 m^{-3} in 2005 and 2007 respectively.

3.2 Soil water content module

The model simulated efficiently the evolution of soil moisture according to the prevailing meteorological conditions, showing an increase of SWC after the first autumn rains and a rapid soil drying at the end of the spring when the drought period started (Figures 3a, b). Spring rain events were more abundant in 2007, amounting 246 mm, while only 80 mm were collected in 2005 (Table 1, Figure 3a). This precipitation distribution allowed a SWC above the wilting point for a longer period during the 2007 growing season.

The average of modelled values of SWC ($\overline{q_{0-15cm}, q_{15-30cm}}$) was better related with TDR field observations at 0-15 cm than modelled $?_{0\text{-}15cm}$ or $?_{15\text{-}30cm}$ separately. RMSE values pointed to similar differences between measured and modelled values in both years (Table 2). The SWC model was able to explain 82 and 77% of the variability of measured values in 2005 and 2007, respectively.

	RMSE		R^2	
Model	**2005**	**2007**	**2005**	**2007**
SWC module	5.1	5.9	0.82	0.77
Pasture growth module		98		0.65
g_s module				
'2005 model'	0.23		0.18	
'2007 model'		0.33		0.32
g_s module MEDPAS Model	0.22	0.33	0.21	0.44
Linear regressions of modelled versus observed g_s values				
'2005 model' and '2007 model'	$y = 0.73\ x + 0.25$; $R^2 = 0.54$; RMSE = 0.28			
MEDPAS Model	$y = 0.83\ x + 0.23$; $R^2 = 0.64$; RMSE = 0.27			

Table 2. Goodness of fit between observed and modelled soil water content (SWC), pasture growth and stomatal conductance (g_s). Stomatal conductance model parameterizations tested: the '2005 model' parameterization was that published in Alonso *et al.* (2007); the '2007 model' parameterization is year-specific, based on field observations of g_s; the MEDPAS model parameterization is common for both years.

3.3 Pasture growth module

Simulated biomass dynamics throughout the 2004/05 and 2006/07 growing seasons followed the prevailing hydro-meteorological conditions (Figure 3a, c). Growth started after the first important autumn rains which filled up the soil water holding capacity and eliminated the limitation imposed by SWC on photosynthesis during the summer (Figure 4). The growth module established the leaf onset on October 24th (DOY 297) in 2004 and October 21st (DOY 294) in 2006. During autumn and winter months, growth was mainly limited by low T (Figure 4) and solar radiation (data not shown), though slightly sunnier conditions during this period allowed a greater growth in 2005 than in 2007 (Table 1; Figure 3c). Early drought conditions in 2005 resulted in an early senescence process due to soil water shortage. The modelled end date of the growing season was set on May 14th (DOY 134) in 2005, while in 2007, mid-spring rain events reduced SWC limitations (Figure 4), allowing the pasture to grow further until July 9th (DOY 190) before the onset of the drought period (Figure 3c). Inter-annual variability in the amount and distribution of precipitation in spring induced differences up to 56 days in the modelled length of the growing period between 2005 and 2007, variation that matched field observations. The growth model was able to explain 65% of the variability of biomass measured values with average deviations of 98 g DM m^{-2} (Table 2).

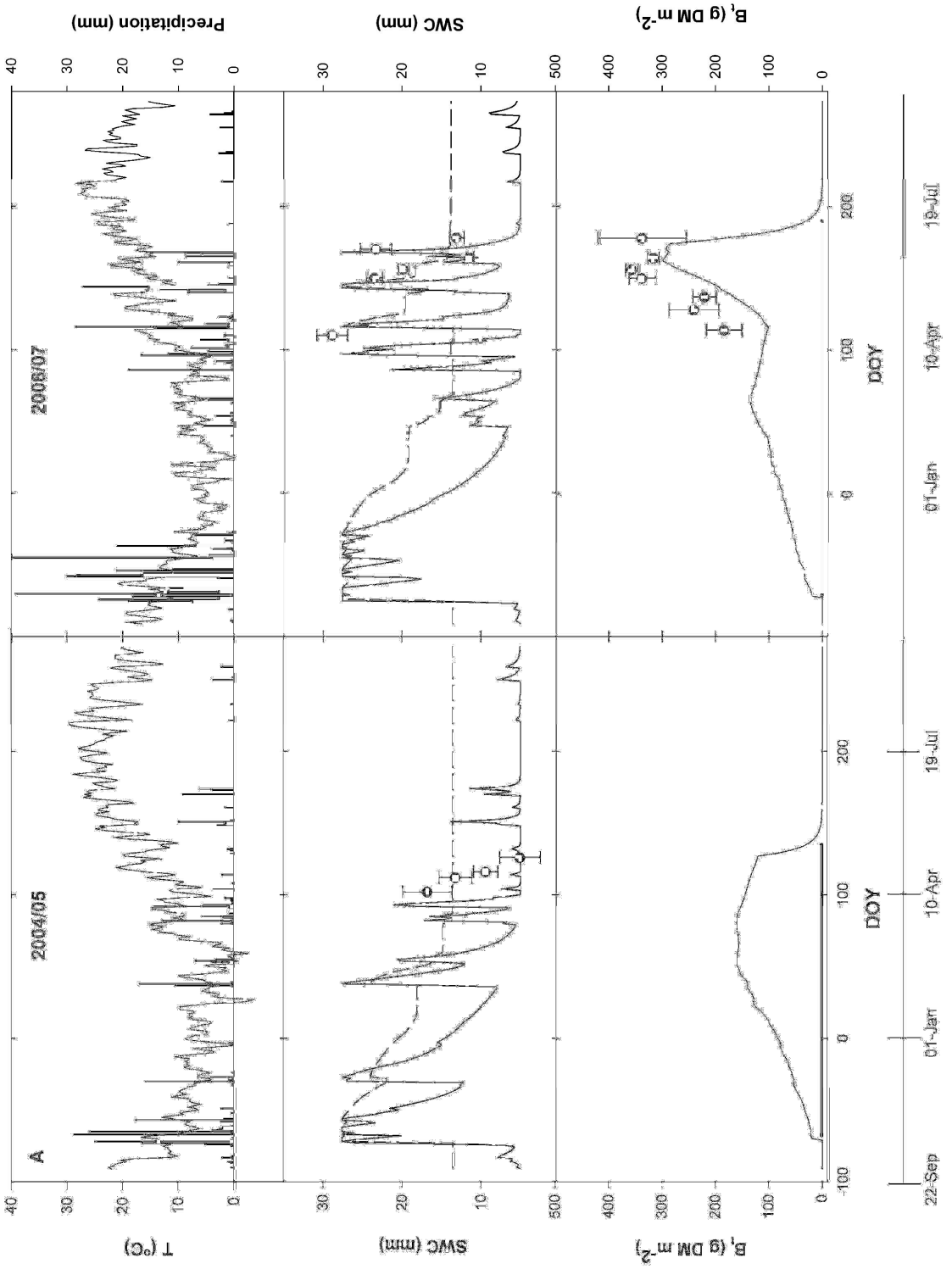

Figure 3. Coupling of meteorology (A), soil water content (SWC) (B) and pasture biomass (B_t) dynamics (C) during the 2004/05 and 2006/07 growing seasons. Temperature is represented in (A) by the solid line and precipitation by vertical bars. Solid and dashed lines in (B) depict modelled SWC (mm) for the 0-15 cm and 15-30 cm depth soil layers respectively. Solid lines in (C) represent the evolution of modelled B_t (g DM m^{-2}). Thick lines in (C) represent the length of the growing season estimated from the growth module. Field measurements of SWC at 0-15 cm depth and B_t are shown as dots (mean $\pm$ SE) in B and C respectively.

3.4 Stomatal conductance modelling

Stomatal conductance measurements of *T. subterraneum* performed in 2007 were used to reparameterize an existing model published in Alonso *et al.* (2007) based on measurements collected in 2005 at the same experimental site. Only g_{max}, f_{phen} and f_{min} required major revisions to fit the 2007 dataset compared to the '2005 model' (Figures 2b and 5d; Table 3). The new f_{phen} function, following the approach adopted in Alonso *et al*. (2007), included a 'plateau' of maximum g_s from the end of April (DOY 110) until May 18th (DOY 138) followed by a quick decline in g_s as the pasture dried out (Figure 5d). The end of the growing season in 2007 was set on July 14th (DOY 195), according with the boundary line fit, resulting in an extension of 45 days compared with measurements on the same species in 2005.

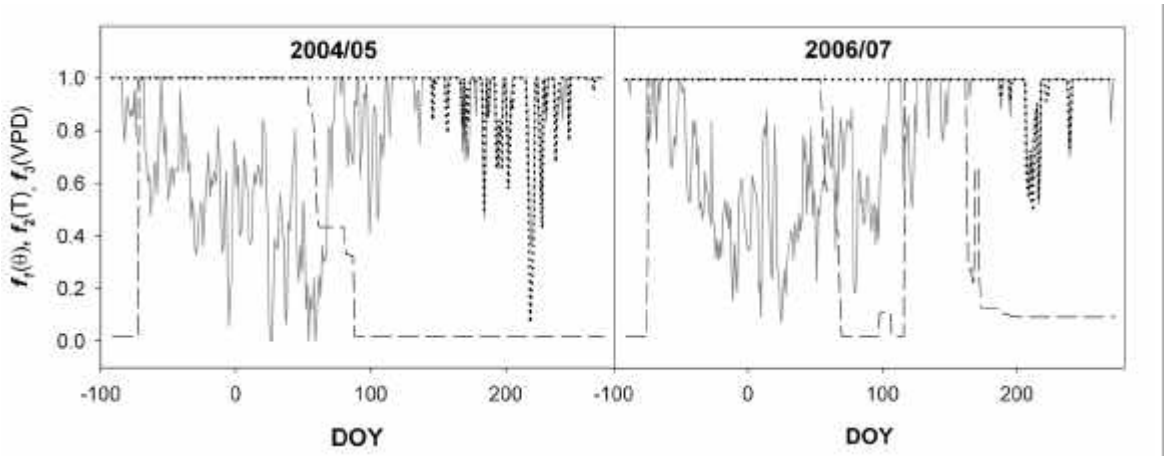

Figure 4. Influence of different environmental factors on biomass growth during two growing seasons 2004/05 and 2006/07. The dashed, solid grey and dotted lines depict the influence of soil water content (f_1(?)), air temperature (f_2(T)) and vapour pressure deficit (f_3(VPD)) on biomass production respectively. Functions vary between 1 and 0, indicating optimum and adverse conditions for biomass production respectively.

On the other hand, the new MEDPAS model included new g_{max}, f_{phen} *(on/off)* and a new f_{SWC} based on 2005 and 2007 g_s data altogether. The new g_{max} matched the higher g_{max} value measured in 2007. The new f_{phen} *(on/off)* established the starting and end of the growing season (on/off function) resulted from the growth module. Since SWC was identified as a mayor driver of g_s, a new f_{SWC} function was added to specifically describe the effects of soil moisture on g_s (Figure 5e).

The parameterization of the MEDPAS model outperformed the specific parameterizations for 2005 and 2007 individually showing higher R^2 values and comparable RMSE values (Table 2). Linear regressions between modelled and observed g_s values indicated that the three parameterizations tended to overestimate low values. However, when using the MEDPAS, the slope of the regression was closer to one and the intercept was lower than with the '2005 model' and '2007 model' (Table 2). Of particular importance for O_3 flux calculations was the fact that the inter-annual variability on g_{max} and length of the growing season were covered by the MEDPAS model using one single parameterization.

	2005 model	**2007 model**	**MEDPAS Model**
g_{max}	0.550	1.18	1.18
f_{min}	0.04	0.02	0.02
T_{min}	8	8	8
T_{opt}	22	22	22
T_{max}	33	33	33
a	0.013	0.013	0.013
VPD_{max}	2.2	2.2	2.2
VPD_{min}	4.3	4.3	4.3
SGS	0	0	
EGS	150	195	
a	0.2	0.2	From growth module:
b	110	110	f_{phen} *on/off*
c	32	45	
D	-	29	
$?_{WP}$	-	-	0.03
$?_{opt}$	-	-	0.188
e	-	-	4.18

Table 3. *Trifolium subterraneum* stomatal conductance (g_s) model parameterizations. The '2005 model' parameterization was that published in Alonso *et al.* (2007). The '2007 model' parameterization is year specific based on field observations of g_s. The MEDPAS model parameterization is common for both years based on field g_s measurements. g_{max}, maximum g_s (mol H_2O m^{-2} s^{-1}); f_{min}, minimum g_s; T_{min}, T_{opt}, T_{max}, minimum, optimum and maximum air temperatures (ºC) for f_T function; VPD_{max} and VPD_{min}, maximum and minimum VPD values (kPa) for f_{VPD}; SGS, start of growing season; EGS, end of growing season; a, minimum f_{phen}; b, number of days from SGS for f_{phen} to reach its maximum; c, number of days during the decline of f_{phen} to again reach its minimum; d, number of days with f_{phen} at its maximum; $?_{WP}$ and $?_{opt}$, minimum and maximum soil water contents (m^3 m^{-3}) for $f_{SWC;}$ e, slope of the decline of f_{SWC} from $?_{opt}$ to $?_{WP}$.

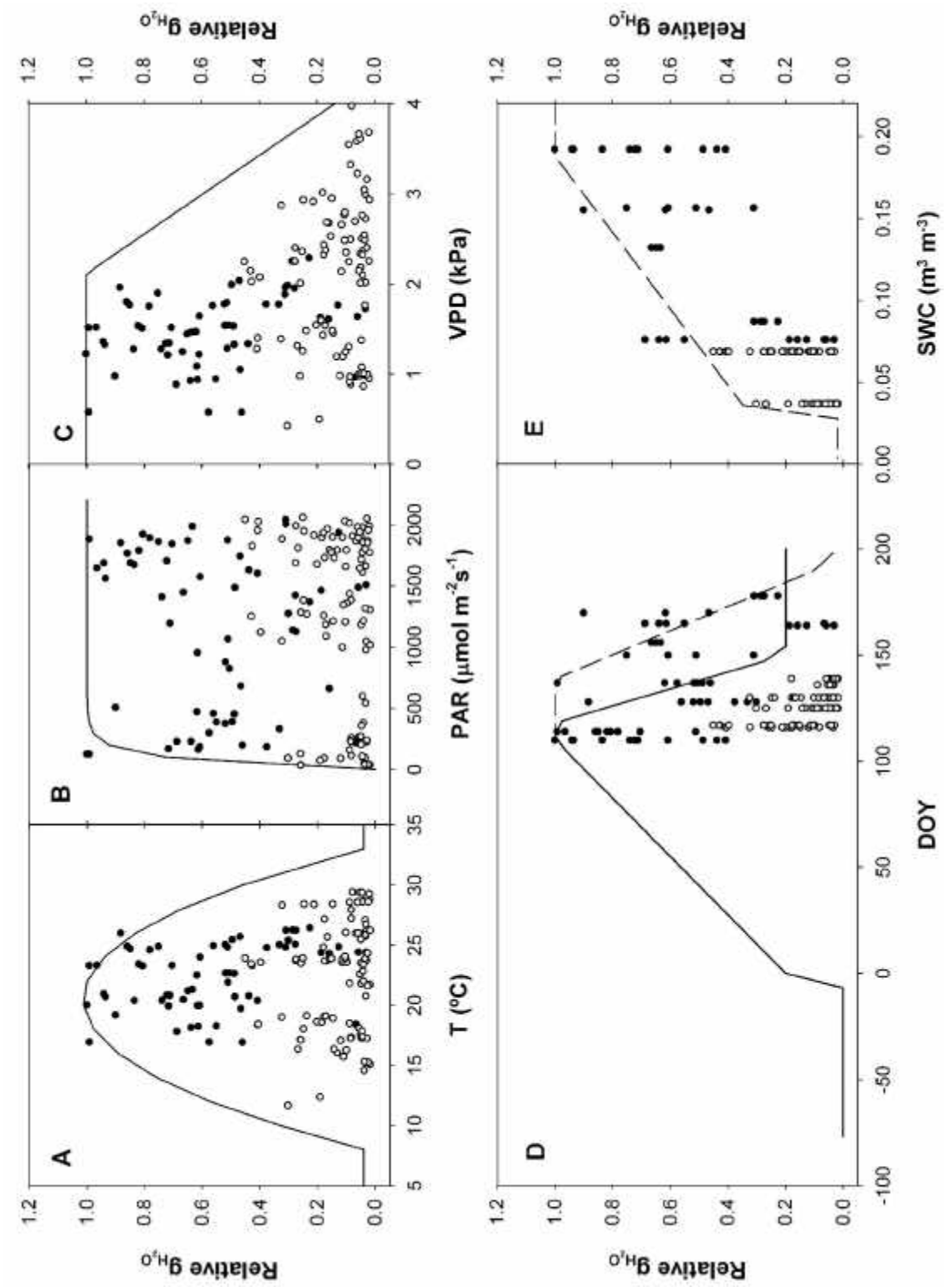

Figure 5. Boundary line analysis underpinning multiplicative stomatal conductance parameterizations for *Trifolium subterraneum* growing in an annual dehesa pasture in central Spain. (A) Air temperature (T); (B) photosynthetic active radiation (PAR); (c) vapour pressure deficit (VPD); (d) plant phenology (day of the year, DOY); (e) soil water content (SWC). Dots depict *T. subterraneum* stomatal conductance (g_s) field measurements collected in 2005 (open dots) and 2007 (closed dots) in a relative scale, where 1 represents full stomatal opening and 0 full stomatal closure. Solid and dashed boundary lines correspond to the 2005 and 2007 parameterizations respectively.

3.5 Accumulated stomatal O_3 fluxes

Accumulated O_3 fluxes absorbed by *T. subterraneum* depended on climatic conditions, O_3 concentrations and the parameterization chosen for g_s estimation. Despite the higher O_3 exposure in 2004/05 during the growing season (AOT40 18912 ppb.h), the MEDPAS model predicted 61% higher accumulated fluxes in 2006/07 (AOT40 6854 ppb.h) in response to the higher g_{max} value and the longer growing season (Figure 6). The three parameterizations showed that $AF_{st}0$ values mainly increased from March 15th (DOY 74) until the end of the growing season in both 2004/05 and 2006/07. $AF_{st}0$ values calculated using the MEDPAS model resembled to those calculated using the year-specific parameterizations ('2005 model' and '2007 model') without the need of adjustments in response to inter-annual variability of the prevailing meteorological conditions. However, big differences in $AF_{st}0$ were found when the alternative year specific parameterization was used (i.e. using the '2007 model' for 2004/05 and the '2005 model' for 2006/07).

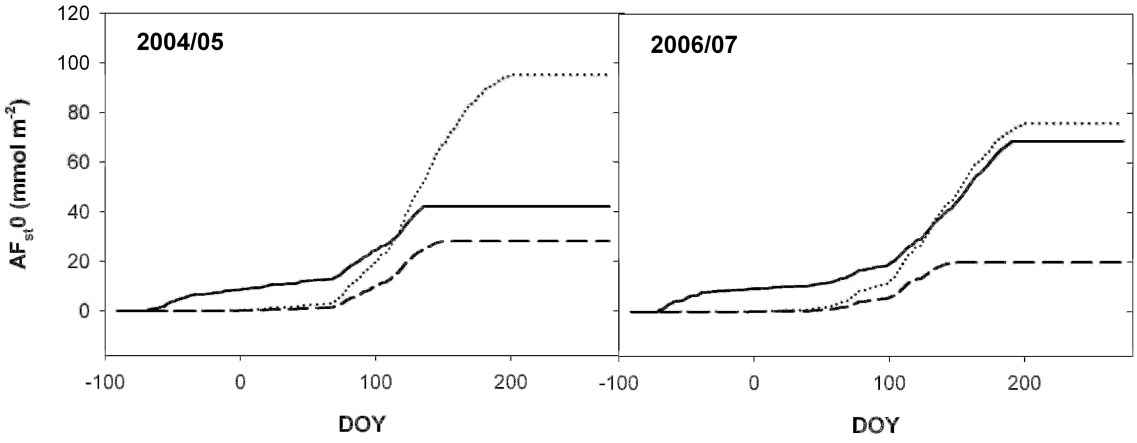

Figure 6. Stomatal ozone accumulated flux ($AF_{st}0$) (mmol m^{-2}) calculated using three different parameterizations of the multiplicative stomatal conductance model. $AF_{st}0$ values were calculated for the prevailing meteorological conditions and ozone concentrations for two growing seasons: 2004/05 and 2006/07. Dashed and dotted lines depict the $AF_{st}0$ calculated using the year-specific 2005 and 2007 parameterizations respectively. The solid line represent the $AF_{st}0$ values derived using the MEDPAS model.

4. Discussion

4.1 MEDPAS model evaluation

The MEDPAS model, coupling modules to estimate SWC, vegetation growth and g_s, reproduced realistically the seasonal and inter-annual variations observed in the field during two contrasting years. The springs of 2005 and 2007 can be considered as representative of dry and humid springs respectively. The MEDPAS model reflected this variability, showing lower $?_{0\text{-}15cm}$, $?_{15\text{-}30cm}$ and growth in 2005 than in 2007, in agreement with the dryer conditions. Modelled variables laid within the range of published values for semi-arid pastures under Mediterranean conditions in both years. Maximum modelled biomass of 160 and 300 g DM m^{-2} in 2005 and 2007 respectively, laid within the range of published values for similar annual grasslands (Xu & Baldocchi, 2004; Aires *et al.*, 2008; Montaldo *et al.*, 2008). However, the model underestimated SWC and B_t by 34% and 31% respectively compared to field observations. The estimation of direct evaporation and SWC could be improved by a better characterization of the depth of the soil evaporation layer (z_e) (Allen *et al.*, 2005b) and the area of bare soil (f_{ew}) from which evaporation occurs preferentially (Allen *et al.*, 1998). The underestimation of biomass production could be partly explained by the underestimated SWC values, but also by the relation of plant response to soil moisture below the superficial layer, considered up to 30 cm depth ($?_{15\text{-}30cm}$). Moreno *et al.* (2005) have reported rooting depths for annual grasslands of dehesa ecosystems up to 80 cm. Similar results have been described for other grassland communities where SWC from the topsoil was not indicative of total plant available soil water, stressing the importance of rooting depth as a factor strongly influencing plant responses to soil water stress (Jäggi *et al.*, 2005). Despite some underestimations, the MEDPAS model reflected the patterns of soil rewetting and drying and the drought onset according with the rain events and ET_c conditions in both years.

Soil moisture availability was the most important factor controlling the length of the growing period. The different rain pattern and soil moisture availability determined a difference up to 56 days in the length of the growing season comparing 2005 and 2007. This different length was mainly related to the date of the end of the growing season. Many species of the Mediterranean region adapt the length of the growing season according to rainfall distribution and soil moisture availability (Peñuelas *et al.*, 2004;

Xu & Baldocchi, 2004; Montaldo *et al.*, 2008). The boundaries of the growing season were efficiently described by the model, reflecting the inter-annual variability of the arrival of the drought season.

Soil moisture availability also determined the g_s dynamics. g_{max} values of two representative annual species found in this community (*T. subterraneum* and *B. hordeaceus*) were 53% lower in 2005 than in 2007 associated with lower SWC values. Therefore, measurements in years with different environmental conditions are needed in order to establish the g_{max} for each species. On the other hand, a whole range of g_{max} values were found comparing families growing within the same pasture in 2005 (Alonso *et al.*, 2007) and 2007. Inter-families variability was equivalent to the inter-annual variability found on just one species. The MEDPAS model outperformed year-specific parameterizations for g_s modelling of *T. subterraneum*. The inclusion of a SWC module and a f_{SWC} in the g_s algorithm improved g_s calculation by 10% and most importantly, explained the inter-annual variability found in g_{max}. The combination of the SWC, growth and g_s modules in the MEDPAS model resulted in a single model parameterization that can be used under the varying climatic conditions common in Mediterranean environments. Year-specific combinations of f_{phen} and g_{max} used in the '2005 and 2007 g_s models' as surrogate for f_{SWC} and f_{phen} *on/off*, accounted for inter-annual differences of stomatal aperture and growing season length, but should be reparameterized under varying environmental conditions resulting in a simpler but unsuitable approach to model g_s.

All g_s parameterizations resulted in overestimations of low gas exchange measurements. This problem has been also found using the multiplicative approach to model g_s for other species (Danielsson *et al.*, 2003; Büker *et al.*, 2007). The overestimation of low g_s values obtained with the multiplicative model has been related to the reduced sensitivity of stomata to VPD during the late afternoon (Elvira *et al.*, 2007), suggesting that some diurnal changes in stomatal function may result from metabolism processes with a circadian rhythm (Chaves *et al.*, 2002) or from a reduction of plant water potential over the course of the day (Pleijel *et al.*, 2007). More mechanistic g_s modelling approaches, using photosynthesis based algorithms, have resulted in improvements of g_s predictions for durum wheat compared with the multiplicative approach (Büker *et al.*, 2007). However, the high input data requirements of parameters difficult to interpolate

spatially may limit its current use for ozone risk assessments in favour of the multiplicative model.

4.2 Ozone stomatal flux calculations for dehesa pasture communities

Efforts have been made to develop a generic modelling approach to simulate seasonal time courses of stomatal O_3 fluxes to grasslands in Europe using a perennial grass, *Lolium perenne*, as a model species (Ashmore *et al.*, 2007). Though this well validated model can be appropriate for perennial grasslands, it could not be applied to dehesa pastures because the annual dynamic includes a seed stage not reflected in the model. These communities avoid the summer drought through the seed stage. Therefore, the high O_3 exposures during the summer should not be accumulated for annual dehesa pastures. However, early spring O_3 doses can be extremely relevant in terms of O_3 sensitivity for dehesa annual communities, since spring is the period when annual plants accumulate reserves for flowering and seed production (Aers *et al.*, 1991). Indeed, deleterious effects on biomass production and forage quality have been reported after short-term O_3 exposures of *T. subterraneum* during the vegetative period (Gimeno *et al.*, 2004a; Sanz *et al.*, 2005)

Modelled stomatal fluxes of O_3 at the leaf level for 2004/05 and 2006/07 were highly variable depending on the g_s model parameterization chosen. The MEDPAS model resulted in accumulated fluxes in 2004/05 and 2006/07 comparable with those calculated using year-specific parameterizations. As a result of higher g_s and longer accumulation period, the MEDPAS model yielded higher $AF_{st}0$ at leaf level in 2006/07 compared with 2004/05, despite the lower AOT40 value recorded in 2007. In both years, AOT40 spring values were well above the critical level of 3000 ppb.h to protect vegetation, set in the European legislation (Directive 2008/50/EC). In 2005 AOT40 was also higher than the AOT40 value reported as phytotoxic for *T. Subterraneum* (Gimeno *et al.*, 2004a; Sanz *et al.*, 2005). Ozone impacts under such high exposures would be expected but, whether lower flux values caused by soil water shortage in 2005 would result in reduced O_3-induced effects is something that needs further investigation. Stable carbon isotope ratios have indicated that, under natural field conditions, lower g_s may not protect plants from O_3 stress in some species (Jäggi *et al.*, 2005). There is a need to clarify the possible interactive effects of O_3 and other abiotic stresses common in the Mediterranean basin such as drought.

The MEDPAS model has been used to calculate absorbed O_3 fluxes at the leaf level in agreement with previous studies on O_3 doses. From an O_3 risk assessment perspective, some limitations have been identified with the use of the leaf level scale. Ashmore *et al.* (2007) found different responses between whole canopy and upper canopy leaves to temperature, management and soil water deficit. Furthermore, it is recognized that flux modelling scale should be related with the nature of the impacts to be assessed (Ashmore *et al.*, 2007). In this regard, the development of canopy stomatal gas exchange measurement techniques seem to be the most appropriate approach for future gas exchange modelling in dehesa pastures. This approach would also avoid the problems arose by using a single species such as *T. subterraneum* for O_3 flux modelling in highly biodiverse pastures like dehesas. Ozone risk assessment considering whole canopy O_3 fluxes and community level responses to these fluxes are questions to be further investigated.

5. Conclusions

The MEDPAS model, coupling the SWC, growth and g_s modules, was able to reflect the inter-annual variability observed in annual pasture dynamics induced by the varying climatic conditions and especially by the amount and distribution of rainfall. Soil water availability was identified as a strong controller of plant dynamics determining the length of the growing period, plant growth and plant stomatal conductance. In this sense, the MEDPAS model can be used to estimate stomatal O_3 fluxes in dehesa annual pastures. However, some limitations were identified when a single species was used to estimate O_3 fluxes in this highly biodiverse pasture. Also refined parameterizations should be developed in the future to describe the response of plants to SWC and to develop canopy scale models following the framework presented in this study. This model constitutes a useful basis to simulate how different components of the global change may affect O_3 risk assessments for dehesa annual pastures under current and future climate scenarios.

Acknowledgements

This study was funded by an agreement between the Spanish Ministry of Environment (MARM) and CIEMAT on ‘‘Critical loads and levels” and by the Spanish project ‘*Spanish Woodlands and Climate Change: Threats and Opportunities*’ (MONTES) – Consolider Ingenio (CSD2008-0040).

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Appendix 1

Equations and parameters used by the SWC and the pasture growth modules, modified from Allen *et al.* (1998) and Montaldo *et al.* (2005, 2008), are presented in Tables A.1 and A.2 respectively. Only parameters modified or selected among several possibilities within each module have been presented. Finally a list of abbreviations used in the SWC and pasture growth modules is provided.

Table A.1. Equations and parameters used in the soil water content module.

Term	Equation/parameter	Source
Potential evapotranspiration (ET_o)	$ET_0 = \frac{\Delta(R_n - G) + 86400\, r_a c_p \frac{VPD}{r_a}}{\Delta + g\,(1 + \frac{r_s}{r_a})}$ $\Delta = f(T)$ $R_n = R_{ns} - R_{nl}$ $R_{ns} = (1 - a')R_s$ $R_{nl} = f(T, e_a, R_s, R_a, z)$ $G \cong 0$ $r_a = f(h)$ $g = f(P_{atm})$ $r_s = \frac{r_l}{LAI_{active}}$ $LAI_{active} = 0.5 LAI_g$	A1998
Actual evapotranspiration (ET_c)	$ET_c = E_i + T_i$ $E_i = K_e ET_0$ $T_i = K_s K_{cb} ET_0 \ (0 - 30\ cm)$	Modified from A1998
Evaporation component (E_i)	$K_e = \min\{(K_r (K_{c\max} - K_{cb})); (f_{ew} K_{c\max})\}$ $K_r = \begin{cases} 1 & if\ D_e < REW \\ \frac{TEW - D_{e-1}}{TEW - REW} & if\ REW \le D_e \le TEW \\ 0 & if\ D_e > TEW \end{cases}$ $f_{ew} = \min\{(1 - f_c); f_w\}$ $f_c = \left(\frac{K_{cb} - K_{c\min}}{K_{c\max} - K_{c\min}} \right)^{(1+0.5h)}$	A1998

Table A1 (cont.)

Transpiration component (T_i)	$K_s = \begin{cases} 1 & if\ D_r < RAW \\ \dfrac{TAW - D_r}{TAW - RAW} & if\ RAW \le D_r \le TAW \\ 0 & if\ D_r > TAW \end{cases}$ $RAW = p \cdot TAW$ $TAW = 1000(q_{FC} - q_{WP}) z_r$ $p = 0.6 + 0.04(5 - ET_c)$ $K_{cb} = K_{c\,\min} + (K_{cb\ full} - K_{c\,\min})(1 - e^{-0.7\,LAI_g})$ $K_{cb\ full} = (1 + 0.1h) + [0.04(u-2) - 0.004(RH_{\min} - 45)]\left(\dfrac{h}{3}\right)^{0.3}$ D_e, D_r *from daily soil water balance*	A1998 A1998
Transpiration from surface layer (0-15 cm)	$T_i = K_{ti}\ K_s\ K_{cb}\ ET_0 \quad (0 - 15\ cm)$ $K_{ti} = \left(\dfrac{1 - \dfrac{D_e}{TAW_{ze}}}{1 - \dfrac{D_r}{TAW_{zr}}}\right)\left(\dfrac{z_e}{z_r}\right)^{0.6}$	Modified from A2005a
Total extractable water	$TEW = 1000(q_{WP} - 0.37 q_{WP}) z_e$	Modified from A1998

Parameters

Albedo	a'	0.23	A1998
Specific heat of air	c_p (MJ kg^{-1} ºC^{-1})	$1.013 \cdot 10^{-3}$	
Leaf stomatal resistance	r_l (s m^{-1})	70	A1998
Canopy height	h (m)	From growth model	
Green LAI	LAI_g (m^2 m^{-2})	From growth model	
Soil water content at field capacity	?$_{FC}$ (m^{3}m^{-3})	0.18	Field
Soil water content at wilting point	?$_{WP}$ (m^{3}m^{-3})	0.09	Field
Rooting depth	z_r (m)	0.30	Mor2005
Minimum K_c value for dry bare soil	$K_{c\,\min}$	0	A2005b
Fraction of soil surface wetted by precipitation	f_{ew}	1	A1998
Readily extractable water by evaporation	REW (mm)	9	A2005b
Depth of soil affected by evaporation	z_e (m)	0.15	A1998

Sources: A1998, Allen *et al.*, 1998; A2005a, Allen *et al.*, 2005a; A2005b, Allen *et al.*, 2005b; Mor2005, Moreno *et al.*, 2005. Field means measured or calibrated in the field.

Table A.2. Equations and parameters used in the annual grasslands growth module.

Term	Equation/parameter	Source
Photosynthesis	$P_g = e_p(PAR)\, f_{PAR} PAR\, f_1(q)\, f_2(T)\, f_3(VPD)$ $e_p(PAR) = a_0 + a_1 PAR + a_2 PAR^2$ $f'_{PAR} = 1 - e^{(-k_e LAI_g)}$ $f_1(q_{15-30}) = \begin{cases} 1 & if\ q > q_{\lim} \\ \dfrac{q - q_{sen}}{q_{\lim} - q_{sen}} & if\ q_{sen} \le q \le q_{\lim} \\ 0 & if\ q > q_{sen} \end{cases}$ $f_2(T) = \begin{cases} 0 & if\ T < T'_{\min}\ or\ T \ge T_{\max} \\ \left(1 - \dfrac{T_{\lim\, low} - T}{T_{\lim\, low} - T'_{\min}}\right) & if\ T'_{\min} \le T < T_{\lim\, low} \\ 1 & if\ T_{\lim\, low} \le T < T_{\lim\, high} \\ \left(\dfrac{T'_{\max} - T}{T'_{\max} - T_{\lim\, high}}\right) & if\ T_{\lim\, high} \le T < T'_{\max} \end{cases}$ $f_3(VPD) = \begin{cases} 1 & if\ VPD < VPD_{\lim} \\ \left(\dfrac{VPD'_{\max} - VPD}{VPD_{\max} - VPD_{\lim}}\right) & if\ VPD_{\lim} \le VPD < VPD_{\max} \\ 0 & if\ VPD \ge VPD_{\max} \end{cases}$	Modified from M2005
Allocation	$a_a = \dfrac{x_a + \Omega l}{1 + \Omega[1 + l - f_1(q)]}$ $l = e^{-k_e LAI_g}$	M2008
Respiration	$R_g = m_a f_4(T) B_g + g_a a_a P_g$ $f_4(T) = Q_{10}^{\frac{Tm}{10}}$	M2008
Senescence	$S_g = d_a B_g$	M2008
Litterfall	$L_a = k_a B_d$	M2008

Table A2 (cont.)

Parameters			
PAR extinction coefficient	k_e	0.58	N2000
Allocation to leaves	$?_a$	0.6	M2008
Allocation parameter	O	0.8	M2008
Maintenance respiration coefficient leaves	m_a (d^{-1})	0.006	M2005
Temperature coefficient in respiration	Q_{10}	2	N2000
Growth respiration coefficient leaves	g_a	0.22	M2008
Growth respiration coefficient roots	g_r	0.1	M2008
Death rate green leaves	d_a (d^{-1})	0.0064 0.14 (senescence)	M2005 N2000
Rate litter production	k_a (d^{-1})	0.001 0.23 (senescence)	Field M2008
Specific leaf area green aerial biomass	c_g (m^2 g DM^{-1})	0.0105	N2000
Specific leaf area death aerial biomass	c_r (m^2 g DM^{-1})	0.02	M2005
Canopy height coefficient	c_h (m g C^{-1})	0.5	Modified from A&B2005
Radiation use efficiency coefficients	a_0	0.0143	From field
	a_1	0.0842	assimilation
	a_2	0.146	rates
Limiting soil moisture	$?_{lim}$ (m^3 m^{-3})	0.11	SWC model
Soil moisture at senescence	$?_{sen}$ (m^3 m^{-3})	0.09	Field
Minimum temperature	T'_{min} (K)	273.15	Field
Lower limit of the optimum temperature	$T_{lim\ low}$ (K)	288.15	Field
Higher limit of the optimum temperature	$T_{lim\ high}$ (K)	299.15	Field
Maximum temperature	T'_{max} (K)	308.15	Field
Limiting VPD	VPD_{lim} (HPa)	25	Field
Maximum VPD	VPD'_{max} (HPa)	45	Field

Sources: A&B2005, Arora & Boer, 2005; M2005, Montaldo *et al.*, 2005; M2008, Montaldo *et al.*, 2008; N2000, Nouvellon *et al.*, 2000. Field means measured or calibrated in the field.

List of abbreviations used in the soil water content and pasture growth modules of MEDPAS model (by alphabetical order)

a_0, a_1, a_2, radiation use efficiency coefficients;
a_a, allocation ratio to aerial biomass;
B_d, standing death aerial biomass (g m^{-2});
$B_{g\,0}$, initial green biomass (g m^{-2});
B_g, green biomass (g m^{-2});
B_{seeds}, seed biomass (g m^{-2});
B_t, total biomass (g m^{-2});
C_L, green biomass (Kg C m^{-2});
c_g, specific leaf area of green aerial biomass (m^2 g DM^{-1});
c_h, canopy heigh coefficient (m g C^{-1});
c_p, specific heat of the air at constant pressure (MJ Kg^{-1} $^oC^{-1}$);
CR_i, soil water capillary rise (mm);
d_a, death rate of green leaves (d^{-1});
D_r, root zone soil water depletion (mm);
D_e, evaporation zone soil water depletion (mm);
DP_i, soil water deep percolation (mm);

e_a, actual vapour pressure (kPa);
E_i, soil water evaporation (mm);
$ET_{c\,i}$, soil water evapotranspiration (mm);
f_1(?), stress function of soil moisture;
f_2(T), stress function of temperature;
f_3(VPD), stress function of vapour pressure deficit ;
$f_4(T_m)$, function representing the effect of temperature on maintenance respiration;
f_c, fraction ground of covered or shaded by plants;
f_{ew}, fraction of exposed and wetted soil;
f'_{PAR}, fraction of PAR absorbed by the canopy;
f_w, fraction of soil surface wetted by precipitation;
G, soil heat flux (MJ m^{-2} d^{-1});
g_a, growth respiration coefficient;
h, canopy height (m);
I_i, irrigation (mm);
Int_i, rainfall interception loss (mm)
k_a, rate of litter production (d^{-1});
K_{cb}, basal crop coefficient;
$K_{cb\,full}$, basal crop coefficient at full ground cover;
$K_{cb\,min}$, minimum K_c for bare soil;
$K_{c\,full}$, value of K_c at full ground cover;
$K_{c\,max}$, maximum value of K_c following rain;
K_e, soil evaporation coefficient;
k_e, PAR extinction coefficient;
K_r, evaporation reduction coefficient;
K_s, water stress transpiration coefficient;
K_{ti}, proportion of soil water extracted by transpiration from the evaporation layer;
LAI_0, initial value of leaf area index (m^2 m^{-2});
LAI_{active}, active leaf area index (m^2 m^{-2});
LAI_g, green leaf area index (m^2 m^{-2});
L_a, litter fall (g m^{-2} d^{-1}) ;
m_a, maintenance respiration coefficient (d^{-1});
p, fraction of TAW extractable before water stress;
P_{atm}, atmospheric pressure (kPa);
PAR, photosynthetically active radiation (KW m^{-2}, µmol m^{-2} s^{-1});
P_i, precipitation (mm);
P_g, rate of carbon gain (g m^{-2} d^{-1})
Q_{10}, temperature coefficient in respiration;
r_a, aerodynamic resistance (s m^{-1});
r_l, leaf stomatal resistance (s m^{-1});
r_s, surface resistance (s m^{-1});
R_a, extraterrestrial ratiation (MJ m^{-2} d^{-1});
RAW, readily available soil water in the root zone (mm);
REW, readily extractable water by evaporation (mm);
R_g, respiration rate of leaves (g m^{-2} d^{-1});
RH_{min}, average minimum daily relative humidity (%);
R_n, net radiation (MJ m^{-2} d^{-1});
R_{nl}, net longwave radiation (MJ m^{-2} d^{-1});
R_{ns}, net solar radiation (MJ m^{-2} d^{-1});
RO_i, run-off (mm);

r_s, bulk surface resistance (s m^{-1});
R_s, Solar radiation (MJ m^{-2} d^{-1}, kW m^{-2});
S_g, senescence rate of green leaves (g m^{-2} d^{-1})
SW_f, average seed weight per family (g);
T, air temperature (ºC, K);
TAW_{ze}, total available soil water for plants in the evaporation zone (mm);
TAW_{zr}, total available soil water for plants in the root zone (mm);
$T_{c\,i}$, soil water plant transpiration (mm);
TEW, total evaporable soil water (mm);
$T_{lim\ low}$, lower limit of optimum temperature (K);
$T_{lim\ high}$, higher limit of optimum temperature (K);
T_m, mean daily temperature (K);
T'_{max}, maximum temperature (K);
T'_{min}, minimum temperature (K);
u, wind speed (m s^{-1});
VPD, vapour pressure deficit (kPa, HPa);
VPD_{lim}, limiting value of VPD (HPa) ;
VPD'_{max}, maximum value of VPD (HPa) ;
z, altitude (m);
z_e, depth of soil affected by evaporation (m);
z_r, rooting depth (m) ;
a', albedo;
?, psychrometric constant (kPa ºC^{-1});
? , slope of the saturation vapour pressure curve;
e_p, radiation use efficiency (g DM kW PAR^{-1});
$?_{FC}$, soil water content at field capacity (m^3 m^{-3});
$?_{i,}$ soil water content (m^3 m^{-3});
$?_{lim}$, limiting soil moisture (m^3 m^{-3});
$?_{sen}$, soil moisture at senescence (m^3 m^{-3});
$?_{WP}$, soil water content at wilting point (m^3 m^{-3});
?, scalar index of the availability of light for the allocation process;
$?_a$, allocation ratio to aerial biomass;
$?_a$, air density (kg m^{-3});
O, allocation parameter;

6. GENERAL DISCUSSION

Ozone (O_3) is a widespread air pollutant whose background levels continue rising steadily in the Northern hemisphere (Vingarzan, 2004). Current ambient levels are known to be high enough to reduce crop yield and to cause significant changes in the composition of wild plant communities in many parts of Europe, North America and Asia (Black *et al.*, 2000; Fuhrer & Booker, 2003; Ashmore, 2005; Bassin *et al.*, 2007; Feng & Kobayashi, 2009). Furthermore, background levels are expected to continue growing in the next decades if measures to control O_3 precursor emissions are not reinforced (Dentener *et al.*, 2006; Royal Society, 2008).

Ozone critical levels to protect vegetation have been derived from relationships between atmospheric O_3 concentrations and plant responses obtained in fumigation experiments (Fuhrer *et al.*, 1997). These critical levels are currently used in the development of effect-based pollution abatement strategies and risk assessments under the UNECE Convention on Long-Range Transboundary Air Pollution (CLRTAP) (Fuhrer & Booker, 2003; Simpson *et al.*, 2007). Moreover, O_3 critical levels have been adopted by the European legislation on air quality (Directive 2008/50/EC). Recently, it has been recognized that O_3-induced effects are more related to stomatal O_3 fluxes absorbed by the vegetation than to atmospheric concentrations (Fuhrer *et al.*, 1997; Ashmore, 2005). Therefore, over the last years, intense efforts have been developed to define new critical levels based on stomatal fluxes. Flux-based indices could account, to some extent, for the influence of climatic and phenological factors on O_3-induced effects on vegetation. The work presented in this thesis contributes to the development of flux-based approaches for establishing O_3 critical levels and risk assessment derivation within the context of the CLRTAP.

Various aspects of modelling stomatal ozone uptake and the derivation of dose – response relationships have been discussed throughout the papers presented in this thesis (chapters 2 – 5). Special attention has been given to three issues involved in the establishment of O_3 dose-response relationships: (1) ozone effects on crops and pastures; (2) stomatal conductance modelling; and (3) comparison between current O_3 exposure-response and dose-response relationships. A general overview of these three subjects will be provided in the next sections, followed by a summary of future research needs

for developing dose-response relationships and the establishment of O_3 critical levels for risk assessment.

1. OZONE EFFECTS ON PRODUCTIVITY AND QUALITY OF CROPS AND SEMI-NATURAL VEGETATION

Under the framework of the CLRTAP, vegetation has been divided into 3 main categories for studying O_3 effects: trees, crops and semi-natural vegetation. Two economical important crops in Europe have been studied in this work, lettuce and winter wheat. However, specific dose–response relationships for O_3 risk assessment and critical levels derivation are lacking. These two crops are widely grown in Europe. Lettuce is mainly produced in southern Europe, where 65% of total production takes place, whereas winter wheat is the most commonly grown crop across Europe. Also O_3 effects on semi-natural vegetation communities have been assessed using mesocosms of ryegrass and white clover, representing a species mixture common in managed pastures for grazing.

Realistic O_3 fumigation in open-top chambers (OTC) resulted in negative effects on the productivity of lettuce and winter wheat. Lettuce and wheat have been classified as O_3-sensitive crops according to the results of previous fumigation experiments (Mills *et al.*, 2007). In this study, wheat yield proved to be more affected by O_3 than lettuce, in agreement with published results (Mills *et al.*, 2007). O_3-induced reductions in productivity have been described for many agricultural and horticultural crops throughout Europe, USA and Asia (Black *et al.*, 2000: Feng & Kobayashi, 2009), covering a range of responses from visible symptoms (important for crops whose market value depends on leaf appearance), to seed or pod weight loss, or reduced marketable fruit yield (Velissariou *et al.*, 1999; Black *et al.*, 2000; Ashmore, 2005). Our results indicated that the combination of O_3 effects on plant physiology resulted in average reductions of 20 and 25% in lettuce and winter wheat yield under average O_3 concentrations of 57 and 41 ppb respectively. These O_3 concentrations are typically found in areas where these crops are commercially grown. The magnitude of O_3 effects on lettuce found in this study was similar to that reported in a review of previous studies (Mills *et al.*, 2007). Exposure-response relationships derived from this review showed a

good agreement, i.e. a similar slope, with that derived in the present study. Ozone induced reductions on winter wheat yield described in our research also showed a good agreement with a meta-analysis of fumigation experiments, where mean O_3 concentrations around 42 ppb induced wheat yield losses ranging from 10 to 20% (Feng *et al.*, 2008).

In contrast with results using crop species, ryegrass/clover mixtures showed no reduction in total biomass yield under elevated O_3 exposure, despite declines of 53% on clover productivity. White clover has been included among the O_3 sensitive forage species while ryegrass was classified as O_3 resistant (Hayes *et al.*, 2007a). Ozone-induced effects on clover in the mesocosms were compensated by the yield of ryegrass, leading to a reduction in the clover fraction. A high proportion of clover biomass is considered a desired property for forage swards, thus the overall effect of O_3 could be considered negative. These results have also been described on a review of O_3 effects on sown model communities, although the magnitude of the response depended on the species combination and genotypes considered (Bassin *et al.*, 2007).

Intra- and inter-specific variability in plant responses to O_3 was evident throughout the OTC experiments. Although lettuce cultivars tested showed a similar response in terms of dry weight loss, different winter wheat genotypes showed a considerably high variability in the response to O_3 fumigation. Ozone-induced yield losses in winter wheat varied by 15% across genotypes. Intra-specific variability in response to O_3 pollution has been described for a number of crop species (Black *et al.*, 2000: Biswas *et al.*, 2008; Feng *et al.*, 2008; Feng & Kobayashi, 2009). However, it is not currently taken into account in the derivation of exposure- or dose-response relationships for critical levels derivation and risk assessment exercises. Inter-specific variability in O_3 sensitivity among herbaceous plants growing in the same community, such as white clover and ryegrass, has been widely acknowledged (Davison & Barnes, 1998; Bassin *et al.*, 2007). Differences in O_3 sensitivity between species may cause shifts in floristic composition, as it has already been described in different communities (Davison & Barnes, 1998; Bassin *et al.*, 2007). The change towards increasing grasses against reductions in more sensitive clover populations, as we found in our study, has also been described in other experiments (Davison & Barnes, 1998; Bassin *et al.*, 2007).

Among the mechanisms that could explain O_3 effects on productivity, there is the observed accelerated leaf senescence induced by O_3 fumigation in lettuce, winter

wheat and white clover. Ozone-induced accelerated senescence has been widely described for wheat (Ojamperä *et al.*, 1992; Gelang *et al.*, 2000; Pleijel *et al.*, 2007). In our OTC experiments, the normal decline of stomatal conductance (g_s) due to phenological development of lettuce and winter wheat was accelerated by O_3 fumigation. The O_3-induced increase in fibre content observed in white clover also points to an acceleration of senescence processes. Increases in the acid detergent fraction of lignines (ADL) associated with decreasing nutritive quality for herbivores, have been related to accelerated senescence in clover species (Sanz *et al.*, 2005; Muntifering *et al.*, 2006). Ozone may also induce reductions in photosynthesis. Reductions of g_s, photosynthetic rates and total chlorophyll content, together with stimulated dark respiration, have been described for many species under high O_3 concentration, including lettuce, wheat and white clover (Calatayud *et al.*, 2002; Crous *et al.*, 2006; Biswas *et al.*, 2008; Feng *et al.*, 2008). In our study, lettuce growing under elevated O_3 levels in controlled environmental conditions showed reduced g_s values compared with the charcoal-filtered air treatment. Both processes, accelerated senescence and reduced photosynthetic rates, limit the availability of assimilates for leaf growth or seed production, resulting in the observed reduced biomass productivity of the species analyzed.

Interestingly, O_3 also affected the quality of crop yield. Although poorly researched, O_3 effects on quality can be significant (Ashmore, 2005) and may determine the end use, the final price and the nutritive value of crops. Changes in crop quality after exposure to O_3 have been previously reported for grapevines, oil seed rape, potato, rice, soybean, tomato, watermelon or wheat (Heagle *et al.*, 1998; Gimeno *et al.*, 1999; Black *et al.*, 2000; Bermejo, 2002; Vorne *et al.*, 2002; Soja *et al.*, 2004; Piikki *et al.*, 2008b). In some cases, reductions of quality would result in additional economic loss to the producer. However, in other cases like potato, O_3 improved tuber quality, offsetting the reductions obtained in productivity (Vorne *et al.*, 2002). In our study, O_3 increased the grain protein concentration (GPC) of winter wheat exposed to elevated O_3. This could be regarded as a positive effect of O_3 exposure since increased protein concentration is considered a grading factor for wheat yield quality (Pleijel *et al.*, 1999). Despite increases in GPC, protein yield was not affected by O_3 fumigation, indicating that the higher GPC was associated with the reduced dilution of proteins by non-protein compounds in the grain. Similar results have been found in other studies (Pleijel *et al.*,

1999; Piikki *et al.,* 2008b). The improvement in grain quality would partially compensate the negative effects on yield, but only in some of the genotypes analyzed.

In ryegrass/clover mesocosms, O_3 reduced the nutritive value of white clover for ruminant species through increases in fibre content and reductions in *in-vitro* digestibility. Similar reductions in the nutritive quality of pasture species have been described in previous studies (Sanz *et al.,* 2005; Bender *et al.,* 2006; Muntifering *et al.*, 2006). Nutritive quality in these species is determined to a considerable extent by the cell-wall constituents that are partially digestible. The amount of these fibres influences the voluntary forage in-take of animals and the digestibility of the plants, thus driving meat and milk production (Krupa *et al.*, 2004; Muntifering *et al.*, 2006). Ozone reduced the nutritive quality of clover, aggravating O_3-induced losses on productivity. Ozone effects on productivity and quality were integrated by the consumable food value (CFV) index (Krupa *et al.*, 2004), which was more strongly related to O_3 fumigation than clover productivity or quality losses assessed individually.

While reductions in biomass productivity or yield may appear as appropriate traits to describe O_3 impacts on some species, other response parameters should be chosen to define critical levels for O_3 risk assessment on other receptors. Despite its importance, O_3 effects on quality are still not considered for risk assessment. New indices, integrating O_3 effects on productivity as well as relevant yield quality traits, such as the CFV, should be considered for improving assessments of O_3 damage on crops and herbaceous wild species.

Several factors might modify plant response to O_3 under field conditions. Interactive effects of different global change drivers such as CO_2 and O_3 atmospheric concentration, temperature or soil moisture among other climatic factors, have not been considered yet under close to field experimental conditions (Long *et al.*, 2006). Besides the environmental conditions, other factors related to agronomic practices can also modify the response to O_3. Cutting and grazing management, inter-specific competition or nutrient availability have been described to modulate the response of plants to O_3 (Davison & Barnes, 1998; Bassin *et al.*, 2007; Sanz *et al.*, 2007). The ability to include these factors for O_3 risk assessment is still limited (Ashmore, 2005). In the present investigation, O_3-induced effects on ryegrass CFV occurred only under low soil-nutrient availability. In contrast, white clover showed a marked O_3-induced depression of CFV irrespective of soil nutrition treatment. On the other hand, O_3 effects on clover were less

important when plants were grown in mesocosms rather than monocultures. The presence of ryegrass reduced clover ozone uptake, likely due to variations of environmental conditions within the denser canopy in the mixed mesocosms. Other authors have described shifts in photosynthetic active radiation (PAR), wind speed or O_3 concentration within dense canopies (Jäggi *et al.*, 2006; Bassin *et al.*, 2007). These results indicate the need to develop experiments considering different stresses and environmental factors that may modulate the response to O_3 while following the normal management practices in the region. Indeed, it has been recognized that there are a range of potential effects of O_3 at the ecosystem level for which the necessary mechanistic understanding is scant (Ashmore, 2005). This limits their inclusion in current O_3 risk assessment methodologies.

Ozone impacts on yield and quality described above were assessed using OTC experiments. OTC may modify the response of plants since differences in environmental conditions between OTC and the ambient air (AA) have been reported (Long *et al.*, 2006; Pleijel *et al.*, 2007; Piikki *et al.*, 2008a). Mean seasonal increases in air temperature of less than 1ºC, 12% reductions in PAR, increases of less than 5% in relative humidity and reductions in leaf boundary layer resistance were recorded during the U.S. National Crop Loss Assessment Network (NCLAN) open-top chamber programme (Heagle *et al.*, 1988). The meaning of the influence of these microclimatic modifications inside the OTC for crop productivity assessment was confounded by site-to-site variation (Heagle *et al.*, 1988). Wheat plants growing in OTC have shown 9% lower O_3 uptake compared to ambient air plots (Piikki *et al.*, 2008a). Other studies have established the mean seasonal ratio of O_3 uptake OTC:AA between 0.89 and 1.04 depending on species and chamber type (Nussbaum & Fuhrer, 2000). The modification of environmental conditions in OTC may have led to reductions of O_3 effects on soybean yield compared with free-air gas concentration enrichment (FACE) experiments (Morgan *et al.*, 2006). On the contrary, Long *et al.* (2006) concluded that elevated CO_2 concentration effects on yield derived from OTC experiments were overestimated compared with FACE experiments. Thus O_3 impacts derived from OTC experiments may appear to be slightly underestimated or overestimated compared with field conditions depending on the species and environmental conditions considered.

The choice of response parameters and species, the absence of data for many receptors and the extrapolation to field conditions are the major uncertainties in defining

critical levels for O_3 effects on crops (Fuhrer *et al.*, 1997). The heterogeneity of plant communities and the number of modifying factors seem to be the most limiting factors for establishing critical levels for semi-natural vegetation (Bassin *et al.*, 2007). Emphasis on O_3 effects on quality together with productivity is needed (Fuhrer & Booker, 2003) for improving O_3 critical level derivation and risk assessment for crops and wild herbaceous vegetation. Impacts must be linked to appropriate indices describing the regime of O_3 exposure or absorbed dose of plants. This implies some limitations and uncertainties that would be discussed in the next sections. For instance, a key component of flux-based O_3 indices is the stomatal control of O_3 diffusion to the leaf interior. Thus stomatal conductance models are needed in order to establish the dose of O_3 that is entering the leaves, as it will be seen in the next section.

2. STOMATAL CONDUCTANCE MODELLING

Modelling O_3 transfer and specifically stomatal O_3 uptake under variable environmental conditions using robust and well-validated dynamic models that can be linked to large-scale photochemical models is one major limitation in the current knowledge on the effects of O_3 on plants (Fuhrer & Booker, 2003). Among the processes involved in O_3 uptake by plants, the regulation of stomatal aperture is the most important one. Thus, models to estimate stomatal conductance (g_s) are needed in order to calculate the amount of O_3 entering the leaves through the stomata.

The CLRTAP approach for g_s modelling (DO_3SE, Deposition of Ozone and Stomatal Exchange) for O_3 risk assessment and critical levels derivation follows the multiplicative model proposed by Jarvis (1976) and modified by Emberson *et al.* (2000a). The multiplicative model is based on the presumption that an initially defined species-specific maximum stomatal conductance (g_{max}) is diminished by non-optimal meteorological conditions and the phenological stage of the targeted plant (Jarvis, 1976; Emberson *et al.*, 2000a). The influence of environmental and physiological factors is based on empirical relationships derived using the boundary line technique. This model has been widely applied on several species of crops, such as spring wheat and potato (Pleijel *et al.*, 2007), wild herbaceous species such as *Lolium perenne* (Ashmore *et al.*,

2007), *Trifolium repens* (Büker, 2008), *Bromus hordeaceus*, *Bromus sterilis* and *Trifolium subterraneum* (Alonso *et al.*, 2007), and trees, like Holm oak (Alonso *et al.*, 2008), Kermes oak (Elvira *et al.*, 2004), Aleppo pine (Elvira *et al.*, 2007), birch, (Uddling *et al.*, 2004), beech or Norway spruce (Karlsson *et al.*, 2004b). Also the CLRTAP has already proposed flux-based O_3 critical levels for wheat, potato, beech and birch based on the multiplicative approach to model g_s (UNECE, 2008).

Previous studies have highlighted the strong dependency of the multiplicative model on g_{max} (Emberson *et al.*, 2000a; Büker *et al.*, 2007). g_{max} is defined as the species-specific maximum gas exchange rate (UNECE, 2008), measured under optimum conditions favouring stomatal aperture. g_{max} is the uppermost important factor for g_s modelling with the multiplicative approach, since this is the parameter corrected by modifying functions, varying from 0 (stomatal closure) to 1 (full stomatal aperture) depending on key environmental variables known to control g_s. Careful analysis of g_{max} derivation was performed for the species considered in this study. Also a critical review of the literature for winter wheat g_{max} derivation was performed. g_{max} derived for lettuce and white clover showed a good agreement with published values in the literature. Similarly, the g_{max} recorded in winter wheat in the present study was not significantly different from the value derived previously for spring wheat. This conclusion was supported by the results obtained with the revision of the literature and was in agreement with the conclusion of Pleijel *et al.* (2007). However, several studies have highlighted the existence of an intra-specific variability in this parameter. Studies reporting measurements of multiple wheat genotypes have shown variations between 58 and 21% (Jiang *et al.*, 2000; Xue *et al.*, 2002). The g_{max} derived for winter wheat genotypes in our study varied by 39%, but this variability was considerably greater when all the genotypes included in the literature review were considered. Also, we reported a year-to-year variation in the maximum rate of gas exchange was reported for two dehesa species, *Trifolium subterraneum* and *Bromus hordeaceus*. Comparisons between dry and humid years yielded differences in g_{max} up to 53% in both species.

The choice of g_{max} affects strongly the accumulated stomatal flux of O_3 ($AF_{st}Y$) on which critical levels derivation and risk assessments are based. Stomatal O_3 flux values derived for winter wheat genotypes growing under the same O_3 exposure showed variations up to 32%, when employing the extreme values of the g_{max} recorded for UK winter wheat genotypes. Since g_{max} values derived from field experiments varied across

genotypes and years and because of its importance for g_s and stomatal flux modelling, g_{max} derivation should be based on robust datasets. These datasets should consider the range of environmental conditions and genotypes found at local or regional scales, in order to provide reliable flux estimates.

Stomatal conductance multiplicative models derived in previous chapters for winter wheat, white clover and subterranean clover were compared with parameterizations already published for the same species. Boundary line fits to key drivers of g_s, such as temperature, PAR or VPD, were similar to those published earlier for spring wheat (Pleijel *et al.*, 2007; UNECE, 2008), white clover (Mills *et al.*, 2002) and subterranean clover (Alonso *et al.*, 2007). This reveals a general good agreement with pre-existing g_s datasets. However, further adjustments to fit the experimental data, accounting for local or year-specific environmental conditions or intra-specific variability, resulted in improved, experiment-specific, parameterizations. Substantial differences were evident when comparing the f_{O3} function (describing O_3-induced decreases in g_s) we obtained for winter wheat and the published f_{O3} function derived for spring wheat (UNECE, 2008), with winter wheat appearing less responsive to increases in O_3 dose than spring wheat. This conclusion is generally consistent with the reported difference in stomatal response to ozone between spring and winter wheat (see review by Feng *et al.*, 2008). There were also marked differences between spring and winter wheat in the f_{phen} function describing changes in g_s associated with plant phenology. Integration of the f_{phen} relationship for winter wheat revealed that this function may change up to 12% the g_s predictions. Similarly, f_{phen} for subterranean clover naturally growing in a dehesa pasture had to be readjusted to account for inter-annual differences found in plant phenology. Due to the higher spring rainfall, the growing season was extended by 56 days in 2007, compared with the drier year 2005. As a result, major changes in f_{phen} compared with the existing model (Alonso *et al.*, 2007) had to be introduced in order to meet the new field measurements. In some cases new functions were introduced. A function representing the influence of time of day on g_s (f_{time}) was included in the lettuce and winter wheat models to simulate the closure of stomata during the afternoon hours. Daily changes in stomatal function may result from metabolism processes with a circadian rhythm (Chaves *et al.*, 2002) or from a reduction of plant water potential over the course of the day (Pleijel *et al.*, 2007). This last approach, previously used in spring wheat by Danielsson *et al.* (2003), was adopted in

preference to the ? VPD function used in the DO_3SE model for spring wheat and potato (Pleijel *et al.*, 2007), since in our experiments ? VPD reduced the R^2 of modelled *versus* measured g_s. This behaviour possibly reflects that g_s was following a circadian rhythm since non-limiting VPD values (1.7 and 0.6 kPa on average for lettuce and wheat respectively) were maintained during the experiment and plants were kept well-watered throughout. In the case of lettuce, the decrease in g_s observed in the afternoon was associated to a circadian rhythm since g_s measurements were performed in closed chambers maintaining constant conditions.

In the work developed in this thesis, g_s multiplicative models have been derived for lettuce, winter wheat, white clover and subterranean clover. The multiplicative algorithm was able to explain between 42 and 64 % of the variability of the g_s measurements in these datasets, with a general trend to overestimate measured values. The variability explained rose up to values between 55 and 72% when hourly averages (the time scale considered in O_3 flux modelling) were considered. Previous studies frequently encountered a similar behaviour of the multiplicative approach, yielding R^2 values between modelled *vs.* measured g_s typically ranging between 23 and 79% (Danielsson *et al.*, 2003; Misson *et al.*, 2004; Büker *et al.*, 2007), correleations that increased when hourly averages or daily time courses were considered (Büker *et al.*, 2007). Several reasons have been identified to explain the relatively low goodness of fit of g_s multiplicative models. Instantaneous g_s measurements are highly variable since the process of stomatal aperture is influenced by the interplay of a big number of environmental and physiological factors and their feedback relationships, which makes predicting g_s using simple approaches rather difficult (Büker *et al.*, 2007). Uncertainty in the performance of the model increases when some variables are introduced as surrogates of non-measured or unknown variables. For instance, using the ? VPD (Pleijel *et al.*, 2007) in lettuce or winter wheat for accounting the reduced g_s observed in the afternoon, or using year-specific g_{max} and phenology function for subterranean clover to account for inter-annual differences in pasture dynamics, may not be efficient enough for g_s modelling.

Intra-specific variability on g_s may also decrease the goodness of fit of the g_s models. In our study, winter wheat showed differences among genotypes in the timing of anthesis. Wheat phenology can be described by the accumulated thermal time (accumulated average daily temperature) (Pleijel *et al.*, 2007), and anthesis is

considered the plant stage when g_s reaches the maximum value. Despite all genotypes were exposed to the same thermal time during the winter wheat experiment, differences were found in the date of anthesis, pointing to intra-specific differences in plant phenology. This was solved by including a 'plateau' in the f_{phen} function of the proposed winter wheat model, accounting for the period of time when g_{max} is recorded for the different genotypes. Furthermore, measured g_{max}, a key parameter of the g_s model, proved to vary within species as well as inter-annually. However, in the multiplicative model derivation, only one g_{max} was chosen for the entire dataset, following the approach for O_3 risk assessment described in UNECE (2008). Overestimations of g_s by the winter wheat g_s model proposed in this study can be attributed to some extent to intra-specific variations in g_{max} and f_{phen}. The reduced predictive capabilities of the model due to genetic/regional variation in g_{max} and other parameters of the multiplicative approach highlight a need to make regional model paramaterisations to improve local ozone risk assessment procedures.

The multiplicative model has been identified as a semi-empirical approach, based on relationships between g_s and environmental drivers derived from field measurements. However, a mechanistic description of g_s behaviour remains unavailable (Misson *et al.*, 2004). Ball *et al.* (1987) proposed a semi-mechanistic model (Ball, Woodrow and Berry, hereafter referred as BWB model) based on the close link between g_s and the net photosynthetic rate (A_n). The multiplicative and the BWB models have been compared for g_s estimation of some forest trees and crop species (Misson *et al.*, 2004; Uddling *et al.*, 2005; Büker *et al.*, 2007). In general terms, the coefficients of determination (R^2) between measured and modelled values were similar in both cases, with the BWB outperforming the multiplicative model in most of the cases and yielding smaller residuals (observed minus modelled g_s). However, for current O_3 risk assesment applications, the higher input data requirements of the BWB model result in an obvious limitation to g_s estimation at a regional scale, even though the BWB provides a more mechanistic approach (Büker *et al.*, 2007). Furthermore, the BWB models are also subjected to uncertainties (Büker et al., 2007): firstly, the inability to account for seasonal variations in g_s due to phenological development is often cited as a problem; secondly, A_n is required as an input parameter and has to be modelled on a regional scale, a process requiring detailed and species-specific plant-physiological input parameters; and finally, under moderate to severe soil water deficits, uncoupling of g_s

and A_n may occur increasing the uncertainties associated with the BWB model (Misson *et al.*, 2004). Despite their limitations, the semi-mechanistic approaches might give some insights on the relationship between O_3 impacts and stomatal flux because it can link ozone and CO_2 uptake to effects on photosynthesis (Büker *et al.*, 2007).

The MEDPAS model proposed in this thesis was derived for g_s modelling in annual dehesa pastures based on the g_s multiplicative model, but including a semi-mechanistic approach for key variables affecting O_3 uptake. This task was achieved by considering the influence of soil water content, described by means of a soil water balance, both on stomatal gas exchange rate and on pasture growth. The pasture growth module estimates the rate of biomass production throughout the year. The plant biomass produced influences in turn the soil water balance, and serves also to define the growing season length for this annual vegetation. Coupling the three modules (soil water balance, plant growth and g_s) allowed covering the observed inter-annual variability of key parameters of the g_s multiplicative model previously described, especially g_{max} and f_{phen}. The multiplicative model parameterizations based on one-year measurements were not appropriate to model g_s under varying climatic conditions typically found in the Mediterranean area. This variability would imply the necessity to reparameterize the g_s model every year, especially g_{max} and f_{phen}, in order to consider the effects of the inter-annual variation of climatic conditions on plant gas exchange. On the contrary, coupling the g_s multiplicative model with semi-mechanistic modules simulating key processes, as we proposed in the MEDPAS model, proved to outperform current algorithms used for g_s estimation. Similar modelling approaches have been developed for perennial species using the grass *Lolium perenne* as a model species (Ashmore *et al.*, 2007). Though this well validated model can be appropriate for perennial grasslands, it could not be applied to dehesa annual pastures. These communities avoid the summer drought through the seed stage. Therefore, the high O_3 exposures during the summer, commonly registered in Mediterranean areas, should not be accumulated for O_3 risk assessment for annual dehesa pastures. On the other hand, considering other factors such as management regime and nitrogen fertilization influence, as in the *Lolium perenne* growth and gas exchange model, should be considered as the next for improving the MEDPAS model.

The multiplicative and the MEDPAS models applied in the present study have been used to calculate absorbed O_3 fluxes at the leaf level, in agreement with previous studies (UNECE, 2008). From an O_3 risk assessment perspective, some limitations have

been identified with the use of leaf level scale models. Ashmore *et al.* (2007) found different responses between whole canopy and upper canopy leaves to temperature, management and soil water deficit in grassland communities. Other studies have reported variations in environmental conditions depending on canopy structure, including shifts in PAR, wind speed and O_3 concentration (Lambers *et al.*, 1998; Jäggi *et al.*, 2006; Bassin *et al.*, 2007). These factors can modify O_3 fluxes and reduce the negative effects of O_3 concentration on short species growing within complex canopies. In this sense, white clover productivity in our study was less affected by O_3 fumigation in mixed mesocosms than in monocultures. Furthermore, it is recognized that flux modelling scale should be related with the nature of the impacts to be assessed (Ashmore *et al.*, 2007). In this regard, the development of canopy stomatal gas exchange modelling and measuring techniques seem to be the most appropriate approach for future gas exchange estimations. These approaches would also avoid the problems arose by using a single species, such as *T. subterraneum* in dehesas, for O_3 flux modelling in highly biodiverse pastures provided that great inter-specific differences in gas exchange parameters may be found. Great inter-specific differences were found comparing g_{max} between plant families and between species within the *Trifolium* genus growing in a dehesa pasture. Differences up to 2.4-fold and as much as 5-fold in g_{max} values, derived for plant families and *Trifolium* species growing in the same pasture, were recorded in the field respectively. Ozone risk assessment considering whole canopy O_3 fluxes and community level responses to these fluxes are questions to be further investigated.

3. EXPOSURE- *versus* DOSE- RESPONSE RELATIONSHIPS

Current O_3 critical levels to protect vegetation under the CLRTAP framework have been derived from the relationships between atmospheric O_3 concentrations and the observed plant responses, obtained mainly using OTC facilities. In the mid 90's, the AOT40 index was adopted for defining O_3 exposure instead of one, 10, 12 or 24 hours average concentrations, recognizing the importance of cumulative exposure approaches (Kärenlampi & Skärby, 1996). The AOT40 index, the accumulated exposure to O_3

above a threshold of 40 ppb during daylight hours, provided a good fit to a range of experimental data (Fuhrer *et al.*, 1997). For this reason, the AOT40 was selected as an appropriate index for establishing O_3 critical levels and policy development. However, a consensus has recently evolved that O_3 phytotoxic effects are more closely related to the amount of pollutant entering the plant through the stomatal pores and reaching the sites of damage within the leaves (Musselman *et al.*, 2006).

In our study, linear exposure-response relationships were derived from experimental data of O_3 effects on lettuce, winter wheat and white clover. O_3 exposure expressed as AOT40 was correlated with O_3-induced reductions on productivity. Ozone exposure explained from 48 to 83% of the observed reductions, depending on species and genotypes. No significant relationship was found between O_3 exposure and ryegrass production. Furthermore, linear fits between AOT40 and seed weight per plant in winter wheat were reanalyzed for genotypes grouped according to their sensitivity to O_3. The grouping of genotypes improved the results obtained using the overall dataset. Indeed, sensitive genotypes yielded the strongest relationship between AOT40 and plant productivity. The observed genotypic variability in the response of plants to O_3 introduced uncertainties in the definition of exposure-response relationships using the AOT40 index. Exposure-response relationships were also derived for O_3 effects on quality of winter wheat grain and white clover as the response parameter. The AOT40 index performed well, with R^2 ranging between 40 and 96%, thus showing that exposure-based indices could also describe O_3 effects on yield quality and forage nutritive value. Other AOTX indices using different thresholds values were tested to examine the best fit of exposure-response relationships for lettuce and white clover productivity. Only slight improvements of up to 8% were obtained for the exposure-response relationships. Therefore, the most extended AOT40 index was chosen to derive exposure-response relationships for these species. The choice of a 40 ppb threshold by the CLRTAP was based on the decision of focusing on that part of the O_3 concentration which is primarily of anthropogenic origin (Fuhrer *et al.*, 1997).

Exposure-response relationships using the AOT40 index have been developed for a number of crops and wild herbaceous species or plant communities (Fuhrer *et al.*, 1997; Bermejo *et al.*, 2002; Bassin *et al.*, 2007; Mills *et al.*, 2007). However, there are concerns in using exposure as an index, since O_3 uptake rate by plants may be decoupled from the time period when the highest concentrations occur (Musselman *et*

al., 2006). For instance, in this study, modelled O_3 stomatal fluxes for subterranean clover growing in a dehesa pasture in central Spain during two contrasting years showed higher uptake fluxes in the year when comparatively lower AOT40s were recorded. This behaviour was explained by the drier conditions limiting g_s and O_3 uptake by plants, occurring at the same time as high O_3 concentrations. Indeed, it has been cautioned that the use of exposure indices may provide an overestimate of vegetation effects, especially under dry conditions (Fuhrer, 1995; Fuhrer *et al.*, 1997; Grünhage *et al.*, 1999). For this reason, efforts have been made to combine exposure indices and limiting variables such as water to predict vegetation effects (Fuhrer, 1995; Karlsson *et al.*, 2004a; Musselman *et al.*, 2006). In this regard, as described in Karlsson *et al.* (2004), modified exposure indices considering a range of thresholds and including PAR and VPD effects on plant response to O_3 were tested in this work using lettuce as a model species. However, very limited improvements were obtained in predicting O_3 effects on biomass production compared with current AOT40 exposure indices. The modified AOT index (mAOT), however, proved to explain reasonably well the appearance of visible injury records in Karlsson *et al.* (2004a). This disparity of results might be caused by the little relevance of the vapour pressure deficit under northern England conditions, where the maximum VPD recorded was 2.9 kPa, non limiting for lettuce g_s according to field measurements. A similar approach was adopted by Fuhrer (1995) considering soil water availability as a factor (f_{water}) modulating the effects predicted by the AOT40.

In the last years, efforts have been focused on estimating stomatal O_3 uptake fluxes and establishing dose-response relationships. In this study, dose-response relationships based on accumulated O_3 stomatal flux indices ($AF_{st}Y$) using a range of thresholds were derived from experimental data using lettuce, winter wheat and white clover. In our study, O_3 doses calculated using species-specific thresholds explained between 48 and 81% of the reduction observed in yield related parameters and between 39 and 92% for quality traits and nutritive value, with fitting varying with species and genotype. Similarly to the AOT40, flux-based indices prefromance improved when only sensitive genotypes were included in the analysis. Karlsson *et al.* (2004b) also found an improvement of flux-based indices for young trees when sensitive and more tolerant species were considered separately. Usually flux indices assume an instantaneous constant threshold value below which no effect of O_3 is considered. This threshold

value is thought to be related with the O_3 detoxification capacity of plants (Ashmore, 2005). However, the choice of a flux threshold above which O_3 stomatal flux is relevant for plant behaviour is not clear yet. Thresholds tested in the OTC experiments presented here proved to have different impacts on fitting dose-response relationships depending on species and genotypes. Ozone effects on lettuce dry weight were best related with fluxes calculated over a threshold of 1 nmol m^{-2} s^{-1}, while winter wheat yield and white clover biomass production were more related with fluxes over thresholds of 14 and 8 nmol m^{-2} s^{-1} respectively. Differences were found also between sensitive and resistant genotypes of winter wheat, the latter showing a slightly stronger relationship with fluxes above a threshold of 16 nmol m^{-2} s^{-1}. Thresholds selected in our study to accumulate fluxes for dose-response functions for lettuce and winter wheat, were higher and lower respectively, than that of 6 nmol m^{-2} s^{-1} derived from the relationships on spring wheat and potato (Pleijel *et al.*, 2007). The threshold chosen for white clover biomass reduction was similar to that of 10 nmol m^{-2} s^{-1} used for short-term critical level derivation for acute O_3 visible injury on subterranean clover (Karlsson *et al.*, 2004a). The disparity between models could be the result of varying intrinsic defence capability in different species and genotypes. Biswas *et al.* (2008) found differences in antioxidant activities among winter wheat cultivars. Flux thresholds as low as 1.6 nmol m^{-2} s^{-1} have been selected for young trees (Karlsson et al., 2007), pointing out that a high inter-specific variability in the threshold value might be expected.

In the investigation presented in this thesis, AOT40 performed equally well than $AF_{st}Y$ in order to predict O_3 effects on yield and quality traits of winter wheat and white clover. Only in some cases, better linear fits were found using the fluxes. For instance, lettuce biomass reductions were strongly related with flux-based indices, improving the fit by 27% compared with the AOT40. Previous studies using young tree species, spring wheat and potato suggest that flux-based indices out-perform AOT40 across experiments in fitting observed O_3-induced effects on vegetation (Karlsson *et al.*, 2007; Pleijel *et al.*, 2007). However this has not always proven to be the case. For example, Karlsson *et al.* (2004b) combining data of different tree species found that AOT40 performed slightly better than cumulative flux in predicting effects. This possibly reflects uncertainties in the parameterization of the underlying model of g_s (Ashmore, 2005) and in the simple representation of detoxification mechanisms through the flux threshold (Musselman *et al.*, 2006).

In addition to the external O_3 concentration, the uptake of O_3 by plants is primarily influenced by g_s, which is strongly dependent on climatic conditions, varying between species and site characteristics, the position of leaves within the canopy as well as leaf and plant age (Matyssek *et al.*, 2004). Since all these factors are not taken into account when using atmospheric air quality standards such as AOT40, flux-based assessments have been suggested for developing future air quality standards (UNECE, 2008). The flux-based approach for O_3 risk assessment presents some advantages compared with exposure-based indices because it constitutes a more mechanistic approach to O_3 injury. Firstly, it depicts a physiologically-relevant representation of the phenology of the plant (Pleijel *et al.*, 2007); secondly, takes into account the year-to-year variation in environmental conditions that may affect the period of maximum O_3 sensitivity (Pleijel *et al.*, 1998) and thirdly, considers environmental variation between sites. However, the current set of flux indices poses some limitations. They use a very simple representation of detoxification mechanisms through an instantaneous flux threshold below which there is no effect. Nevertheless, detoxification is a dynamic process that cannot be represented by a constant value, since detoxification capacity changes diurnally as well as seasonally (Alonso *et a*l, 1999, 2001; Musselman *et al.*, 2006; Heath *et al.*, 2009). Massman (2004) proposed a conceptual model linking the capacity of defense mechanisms to detoxify the incoming ozone flux with the canopy assimilation rate. The effective dose was calculated as the difference between O_3 uptake and plant detoxification, highlighting the importance of plant defenses (Massman, 2004). The characterization and modelling of plant detoxification capacity is still a challenge for future improvement of the flux-based approaches.

On the other hand, up-scaling methodologies to relate O_3 stomatal fluxes and effects at the leaf level with canopy scale processes have to be refined (Fuhrer & Booker, 2003). Ozone uptake is usually estimated for individual leaves or shoots, but reassurance is needed that leaf scale fluxes are representative of the canopy as a whole (Massman, 2004; Matyssek *et al.*, 2004). Furthermore, to apply O_3 flux models for risk assessment on large scales, uncertainties in the input data for environmental conditions such as soil moisture, leaf area index or wind speed may be a major limitation to model performance (Fuhrer & Booker, 2003).

4. FUTURE RESEARCH NEEDS

Several limitations and uncertainties related to the use of dose-response relationships have been identified throughout the studies presented in the previous chapters and in this discussion. These limitations helped to identify several fields that require further research for developing flux-based ozone critical levels and risk assessments on crops and wild herbaceous species.

It has been recognized that further study of the different O_3 sensitivity among plant species and cultivars will aid to improve O_3 risk assessment under current and future O_3 levels. Ozone critical level derivation should be based on the most sensitive species or genotype identified.

Despite its economic importance, O_3 impacts on yield quality and forage nutritive value are still not considered as response parameters for risk assessments. Plant response parameters, integrating O_3 effects on productivity as well as relevant quality traits, such as the CFV, should be developed for improving assessments of O_3 damage on crops and herbaceous wild species.

There is still a need to understand how different stresses and environmental factors may modulate plant responses to O_3, when following normal management practices in the region. Furthermore, our ability to predict the effects of potential changes in ozone exposure in the context of other factors associated with global change is still limited.

The current approach for calculating effective doses poses also some limitations. Models linking the capacity of defence mechanisms to detoxify the incoming ozone flux are required to derive an effective ozone dose that can be related with O_3-induced damage.

Other limitations of using flux indices are related with the underlying g_s modelling approach. Modelling g_s under variable environmental conditions should be based on robust and well-validated dynamic algorithms that can be linked to large-scale photochemical models. Careful consideration should be given to g_{max} derivation if the multiplicative model is chosen to estimate g_s. This choice, again, should be based on robust datasets, considering the range of environmental conditions and genotypes found local or regionally in order to provide reliable flux estimates.

More mechanistic approaches, such as the BWB model, might be preferred in the future since they give some insights on the effects of O_3 stomatal flux and CO_2 uptake on photosynthesis. However, the higher input data requirements of the BWB model result in an obvious limitation to current g_s modelling at a regional scale. Alternatively, the g_s multiplicative algorithm could also be linked to other semi-mechanistic models describing key variables yielding to more dynamic approaches readily applicable but still poorly developed.

Up-scaling methodologies to relate stomatal O_3 fluxes and effects at the leaf level with canopy scale processes have to be refined. The development of canopy stomatal gas exchange modelling and measuring techniques seem to be the most appropriate approach for future gas exchange modelling. Ozone risk assessment considering whole canopy O_3 fluxes and defining community level responses to these fluxes are questions to be further investigated.

Finally, a general problem related with flux modelling is the limited validation of the models and the extrapolation to field conditions. The latter is still one of the major uncertainties in defining critical levels for O_3 effects on vegetation.

7. CONCLUSIONS

The main conclusions presented in this thesis are summarized below:

1. Ozone (O_3) exposure resulted in negative effects on the productivity of lettuce, winter wheat – crops of great economic value in Europe – and white clover – a valuable species in productive pastures. Quality related traits such as protein content, forage nutritive value or pasture species composition were also affected by O_3, potentially modifying the importance of O_3–induced effects on crops and pastures. The derivation of exposure- or dose-based relationships using reductions on productivity alone may underestimate the effects of O_3 pollution. Thus, other relevant plant response parameters, besides biomass productivity, should be considered as well for establishing O_3 exposure- or dose-based relationships.

2. Plant response to O_3 showed a great intra- and inter-specific variability. The response of different winter wheat genotypes to O_3 fumigation varied up to 15% in terms of yield loss. Furthermore, this response can be modified by factors such as nutrient availability and inter-specific competition. Inter-specific differences on O_3 sensitivity in ryegrass/clover swards resulted in increased ryegrass:clover ratios under O_3 fumigation, with potential consequences for animal feeding and ecosystem functioning. This variability in O_3 sensitivity should be considered when assessing O_3 impacts on local or regional scales.

3. Unexpectedly, exposure-based and dose-based O_3 indices performed similarly in explaining O_3-induced effects on plants. Uncertainties related with the underlying stomatal conductance modelling and the use of fixed thresholds to describe a dynamic process such as O_3 detoxification might be limiting the effectiveness of dose-based indices.

4. Stomatal conductance modelling using the multiplicative algorithm adopted by the Convention on Long-Range Transboundary Air Pollution (CLRTAP) for estimating stomatal O_3 uptake fluxes, proved to be a good approach to simulate g_s for crops and pastures, although a general trend to overestimate measured

values was detected. Parameterizations developed in this study compared well with pre-existing g_s models for the species tested, although fine tuning was needed in some cases. For instance, some modifications needed to be included for modelling stomatal conductance in winter wheat compared to spring wheat, the species currently used to map O_3 risk to cereal yield across Europe under the CLRTAP framework. Although a critical analysis of the available literature revealed no difference between winter wheat and spring wheat maximum stomatal conductance (g_{max}), the key parameter of the multiplicative model, new functions describing changes in stomatal conductance depending on O_3 exposure or plant phenology were included for winter wheat modelling.

5. Significant genotypic variability was evident in the development of the multiplicative model for stomatal conductance modelling. The intra-specific variation observed in g_{max} and phenology highlights uncertainties for the development of ozone risk assessment exercises at European and worldwide scales. This illustrates the need for developing regionalized risk assessment approaches applicable to local crops and conditions.

6. Mediterranean climate, characterized by a high inter-annual variability in meteorological conditions, modulates annual pasture dynamics across years. Soil water availability was identified as a strong driver of plant dynamics, determining the length of the growing period, plant growth and stomatal conductance. A new semi-mechanistic MEDiterranean PASture (MEDPAS) model is proposed for modelling stomatal conductance in annual communities. The MEDPAS model, coupling soil water balance, plant growth and stomatal conductance modules, was able to reflect the inter-annual variability observed in annual pasture dynamics. The MEDPAS model outperformed previous approaches for modelling stomatal conductance and therefore, it should be preferred to estimate stomatal O_3 uptake fluxes in annual communities growing under Mediterranean conditions.

CONCLUSIONES

Entre los resultados más importantes obtenidos en este trabajo se pueden extraer las siguientes conclusiones:

1. La exposición al ozono (O_3) provocó efectos negativos en la productividad de la lechuga, el trigo de invierno – cultivos de gran valor económico en Europa – y el trébol blanco – una especie beneficiosa en los pastos para forraje. Algunos parámetros relacionados con la calidad de la producción, como son el contenido en proteínas, el valor nutritivo del pasto o la composición de especies del pastizal, también fueron afectados por el ozono, lo que podría modular la importancia de los efectos del O_3 sobre los cultivos y los pastos. El establecimiento de relaciones entre la exposición o dosis absorbida y la respuesta de la planta basadas sólo en las pérdidas de producción puede subestimar los efectos del ozono. Por esta razón, deberían considerarse otros parámetros relevantes además de la productividad para el establecimiento de las relaciones de exposición y dosis- respuesta.
2. La respuesta de las plantas frente al O_3 mostró una elevada variabilidad intra- e interespecífica. Las pérdidas de producción obtenidas con diferentes genotipos de trigo de invierno expuestos a una misma fumigación con O_3 variaron hasta en un 15%. Además, se observó que esta respuesta podía ser modulada por diversos factores como la disponibilidad de nutrientes o la competencia interespecífica. La diferente sensibilidad frente al O_3 en pastos de raigrás y trébol provocó que la fumigación con O_3 aumentara la proporción de gramíneas frente al trébol, lo que podría tener consecuencias para la alimentación de los herbívoros y el funcionamiento de los ecosistemas. La variabilidad intra- e interespecífica encontrada en la respuesta frente al O_3 debe ser considerada a la hora de evaluar los posibles impactos del O_3 a escalas local y regional.
3. Contrario a lo esperado, los índices basados en la exposición y en la dosis de O_3 mostraron resultados similares a la hora de explicar los efectos del O_3 sobre la vegetación. Las incertidumbres relacionadas con el modelo de conductancia estomática y la utilización de valores fijos para describir procesos dinámicos

como el caso de los procesos de detoxificación, podrían estar limitando la efectividad de los índices basados en la dosis acumulada.

4. La estimación de la conductancia estomática utilizando el modelo multiplicativo adoptado por el Convenio de Ginebra (CLRTAP) para el cálculo de los flujos absorbidos de O_3, simuló correctamente los valores de conductancia estomática observados en especies de cultivos y pastos, aunque se detectó una tendencia general a sobreestimar las observaciones. Las parametrizaciones desarrolladas durante este estudio fueron similares a las publicadas para estas mismas especies (lechuga, trigo, trébol), aunque en algunos casos fueron necesarios ciertos ajustes. Este fue el caso del modelo elaborado para el trigo de invierno cuando se comparó con el del trigo de primavera, que es la especie que se utiliza en la actualidad para evaluar los riesgos causados por el O_3 sobre los cereales a escala europea en el marco del Convenio de Ginebra. Aunque la revisión bibliográfica efectuada no encontró diferencias entre los trigos de primavera e invierno en el valor de conductancia estomática máxima (g_{max}), un parámetro clave del modelo multiplicativo, sí fue necesario modificar para el trigo de invierno las funciones que describen los variaciones que se observan en la conductancia estomática debidas a los cambios fenológicos y a la exposición al ozono.

5. Se encontró una gran variabilidad entre diferentes genotipos de una misma especie en los parámetros que considera el modelo multiplicativo para el cálculo de la conductancia estomática. La variabilidad intraespecifica observada en los valores de g_{max} y en la fenología pone de relieve la existencia de incertidumbres a la hora de realizar evaluaciones del riesgo de daños causados por el O_3 a escala europea y mundial. Para realizar análisis de riesgos a una escala más regional, es necesario desarrollar metodologías adaptadas a los cultivos característicos y las condiciones locales de cada zona.

6. El clima mediterráneo, caracterizado por una elevada variabilidad meteorológica interanual, regula la dinámica de los pastos anules. La disponibilidad de agua en el suelo fue el factor más importante para explicar la dinámica observada en la vegetación, determinando la duración del periodo de crecimiento, el crecimiento de la vegetación y la conductancia estomática del pasto. Para poder reproducir esta dinámica observada en campo, se propuso un nuevo modelo denominado MEDPAS (*MEDiterranean PASture*) para estimar los valores de conductancia

estomática en pastizales anuales. El modelo MEDPAS, mediante el acoplamiento de tres módulos que describen el balance de agua en el suelo, el crecimiento de la vegetación y la conductancia estomática, fue capaz de reflejar la variabilidad interanual observada en la dinámica de los pastizales. El modelo MEDPAS mejoró los modelos previos existentes para la estimación de la conductancia estomática, por lo este tipo de aproximaciones debería ser utilizado para el cálculo de los flujos de O_3 absorbidos por los pastizales anuales en condiciones mediterráneas.

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