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Fungal-mediated mortality explains the different effects of dung leachates on the germination response of grazing increaser and decreaser species

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ABSTRACT

Depending on their response to grazing, grassland species can be categorized as grazing increasers or decreasers. Grazing by livestock includes several different activities that can impact species differently. Recent evidence suggest that one of these actions, dung deposition, can reduce the germinative performance of decreaser species, thus favouring increasers. The present study tested the hypothesis that decreased germinative success of decreaser species is caused by a greater activity of fungal pathogens under the influence of dung leachates. We performed a phytotron experiment analysing the germination and fungal infections of fourteen species from Mediterranean grasslands. Species were grouped into phylogenetically-related pairs, composed of an increaser and a decreaser species. Seeds of each species were germinated under four different treatments (control, dung leachate addition, fungicide addition and dung leachate and fungicide addition), and the differences in germination percentage, germination speed and infection rate between each increaser species and its decreaser counterpart were analysed. Decreaser species were more affected by mortality than increaser ones, and these differences were higher under the presence of dung leachates. The differences in germinative performance after excluding the effect of seed mortality did not differ between treatments, showing that the main mechanism by which dung leachates favour increaser species is through increased mortality of the seeds of decreaser species. Drastic reductions in the number of dead seeds in the treatments including fungicide addition further revealed that fungal pathogens are responsible for these differences between species with different grazing response. The different vulnerabilities of increaser and decreaser species to the increased activity of fungal pathogens under the presence of dung leachates seems the main reason behind the differential effect of these leachates on species with different grazing response.

Keywords: Germination, Grasslands, Herbivory, Dung, Pathogen, Fungicide.

1. INTRODUCTION

Grazing by domestic livestock has substantial effects on the structure and composition of herbaceous plant communities (Noy-Meir et al., 1989; Peco et al., 2005). Depending on their response to grazing in terms of presence or abundance, several grassland species are categorized as grazing increasers or decreasers (Vesk and Westoby, 2002). Although grazing activity is generally perceived as a single action, it is composed of different activities, such as defoliation, trampling and faeces and urine deposition (Dobarro et al., 2013). Each component of livestock activity can potentially have a specific impact on different plant species, ultimately leading to differences in the relative abundances of many species between grazed and non-grazed areas (Del-Val and Crawley, 2005; Díaz et al., 2007; Kohler et al., 2006, 2004). A deeper understanding of the mechanisms that lead to the different responses of plant species under the different components of grazing is an essential step to understand and predict the consequences that changes in grazing regimes have on grassland diversity.

Among the components of livestock activity, defoliation and trampling seem to be the ones with the greatest impacts (Dobarro et al., 2013; Kohler et al., 2006, 2004). In accordance, a majority of the research aiming at characterizing the mechanisms leading to the different grazing responses of increaser and decreaser species has focused on the effects of defoliation. Experimental evidence suggests that increaser species have a greater tolerance to defoliation (Del-Val & Crawley, 2004, 2005) and higher relative growth rates (Leoni et al., 2009) than decreaser species. However, although the reproductive stage is often neglected when studying differences in grazing response between species, germination and seedling establishment are fundamental determinants of the grassland specific composition with a great abundance of annual plants, such as Mediterranean ones (Espigares and Peco, 1995; Marañón, 1998; Peco et al., 2009). Therefore, studying the effects of grazing on the germination and establishment stages may provide clues about the influence of livestock in Mediterranean grasslands.

Recent studies have shown that the seeds of grazing increaser and decreaser species respond differently under conditions associated to grazing activities. For example, the germination rates of increaser species are reduced when the spectral composition of light indicates the presence of potential competitors (Dobarro et al.,

2010). In addition, Carmona et al. (2013) showed that the proportion of decreaser species seeds that germinate, as well as their germination speed, declined under the presence of dung leachates. By contrast, the seeds of increaser species were not affected by dung leachates (Carmona et al., 2013). Most importantly, changes in germination were not proportional to leachate concentrations, but rather displayed marked thresholds, suggesting that the effect of dung leachates on germination did not depend on its concentration but on its mere presence or absence (Carmona et al., 2013). Such a response was apparently consistent with greater pathogen activity in the leachates environment, which would affect the two kinds of species in different ways.

Fungi are considered as the most important pathogens for seeds (Baskin and Baskin, 2001; Kirkpatrick and Bazzaz, 1979; Kremer, 1993), reducing their survival and germination rates (Blaney and Kotanen, 2001; Crist and Friese, 1993; Schafer and Kotanen, 2004). The abundance and composition of fungal communities is context-dependent; several factors affect soil fungi, including soil temperature, moisture or litter characteristics (Dalling et al., 2011; Mordecai, 2012; Ruprecht et al., 2008; Schafer and Kotanen, 2003). Among these factors, grazing by large herbivores increases the biomass and affects the composition of soil fungal communities (Bardgett and Leemans, 1997; Jirout et al., 2011). Further, not all plant species are equally sensitive to fungal attacks (Leishman et al., 2000; Orrock and Damschen, 2005), a feature that can eventually translate into important differences in their abundances and distributions (Gallery et al., 2010). In this context, the combination of grazing-mediated changes in fungal communities and species-specific seed susceptibilities to fungal attack may be one of the determinants of the differences in composition of grazed and ungrazed sites.

In this paper, we present an experiment aimed to discern whether the different effects of dung leachates on the germination response (germination percentage and speed) of increaser and decreaser species described in Carmona et al. (2013) are caused by different susceptibilities to fungal pathogens. Because phylogeny can influence the grazing response and susceptibility to fungal attack, we select pairs of confamilial species with contrasting responses to grazing. Specifically, we hypothesize that the rate of fungal infections on the seeds of grazing decreaser species will be higher under a treatment of dung leachates, whereas increaser species will be less susceptible to these conditions. If our hypothesis is correct, differences in seed

fungal infections between species with different grazing responses will increase in favour of increasers under the presence of cattle dung leachates, but this increase will be largely reduced or eliminated with the addition of fungicide. Finally, if fungal attacks merely affect seed viability, but not the germination response of the surviving seeds, the differences observed in Carmona et al. (2013) should disappear once that seed mortality due to fungal attacks is taken into account.

2. MATERIAL AND METHODS

In July 2010, we collected seeds of 14 herbaceous species belonging to five different families in Mediterranean grasslands situated 35 km North of Madrid, Spain (40º43´N, 3º43`W, zone description in Peco et al. 2006). We selected the same species used in a previous study that found differences in the effects of cattle dung leachates on the germination of seeds of increaser and decreaser species (Carmona et al., 2013). To control for the effect of phylogeny, species were organized into 4 confamilial and 3 congeneric pairs, each one containing a grazing increaser and a grazing decreaser species (Carmona et al., 2013): *Brassica barrelieri* (L.) Janka paired with *Alyssum granatense* Boiss. and Reuter (*Brassicaceae*); *Spergularia purpurea* (Pers.) D. Don with *Silene scabriflora* Brot. (*Caryophyllaceae*); *Astragalus pelecinus* (L.) Barneby with *Vicia lathyroides* L. (*Fabaceae*); *Poa annua* L. with *Micropyrum tenellum* (L.) Link (*Poaceae*); *Trifolium glomeratum* L. with *Trifolium strictum* L (*Fabaceae*); *Plantago coronopus* L. with *Plantago lanceolata* L. (*Plantaginaceae*); and *Vulpia muralis* with *Vulpia ciliata* Dumort (*Poaceae*). The classification of species to each grazing response group was made according to abundance data from grazed and ungrazed plots in the same area (Carmona et al., 2015, 2012; Peco et al., 2006, 2005). Only species with significant differences between grazed and ungrazed plots and present in more that 10% of the plots were used (see Appendix 1 for a more detailed description of the methods used for this classification).

In January 2011, we collected 3 kg of fresh cattle dung from 30 different dung pats from the area where seeds were collected. After two weeks of drying in a greenhouse, the dung samples were cut-up in small pieces, and thoroughly mixed together. Then, we crumbled the dry dung and recorded its weight. After that, we added three litres of distilled water per kilogram of dung and placed the resulting mixture in a 9 cm diameter plunger coffee-maker and applied a 0.2 Kg/cm^2 pressure for 30 seconds, obtaining a highly concentrated dung leachate. The proportion of

water added is similar to that of fresh dung (Dickinson et al., 1981) and therefore, we expect this leachate concentration to be equivalent to the maximal concentration of dung leachates under field conditions. Finally, we diluted this leachate adding 2.5 parts of distilled water for each part of dung leachate in order to get a leachate concentration similar to the 10% concentration of Carmona et al. (2013). This leachate concentration maximizes differences in germination and seedling development between species with differing responses to grazing (Carmona et al., 2013), and is therefore optimal to analyse the effect of dung leachates on germination.

To test for the differential effects of fungi on increaser and decreaser species, we produced four different treatments: (1) Excrements treatment (E) consisting in the dung leachate described above; (2) Excrements and Fungicide treatment (EF) consisting in the addition of the 1.5 g of the fungicide BELPRON C-50 to each 100 g of the dung leachate; (3) a Control (C) consisting in distilled water; finally, (4) a water and Fungicide treatment (CF), consisting in a 1.5% w/w dilution of fungicide in distilled water, was used in order to detect any possible deleterious effect of fungicide on the measured variables. Any significant difference between the C and CF treatment would indicate such an effect, compromising the validity of the results. The active ingredient of the fungicide is CAPTAN (50% by weight), which is known to be very effective against seed-rotting fungi (Mitschunas et al., 2009; Neergaard, 1977) and has been used in similar concentrations in experiments testing the effects of fungal pathogens on seeds (Blaney and Kotanen, 2001).

We placed 25 seeds per species in 5 cm diameter Petri dishes, over 1.11 g of vermiculite and filter paper. We located the dishes in aluminium trays (each tray containing a dish for each species) and applied 6 ml of the same treatment to all the dishes in the same tray. There were 6 replicates of each treatment for each species, resulting in a total of 24 trays and 336 dishes. These trays were randomly placed in two germination chambers (V-450, ASL S.A. Ibercex, Madrid, Spain) and kept in a 12 h (20 ºC)–12 h (10 ºC) light/darkness and temperature regime, similar to the conditions expected in the study area during the autumn, when germination takes place. We daily monitored the moisture level in the trays and added more distilled water when necessary to avoid desiccation.

During the next six weeks we daily monitored the number of germinated seeds in each dish. We also monitored the number of seeds with visible fungi infections and

moved those seeds to a different dish with the same treatment. Every day, trays were randomly relocated between and within the germination chambers. At the end of the experiment, we calculated for each dish the number of germinated and infected seeds, as well as T50 (days until 50% of germinations). We also performed a pressure test in non-germinated and infected seeds to determine whether those seeds were dead (soft seeds) or alive (hard seeds).

2.1 Statistical analysis

To isolate the consequences of viability losses due to infected seeds from other potential effects of the treatments on germination, we estimated the % of germination of each dish as the number of germinated seeds divided by the initial total seed number placed in the dish minus the number of infected and dead (soft to touch) seeds (GP = number of germinated seeds / [total number of seeds - infected seeds - dead seeds]). Given that the main objective of this study is to analyse the way that the treatments influenced the differences on the germinative response and infection rate between increaser and decreaser species (Carmona et al., 2013), we calculated for each tray the difference in % germination (DGP) between the species of each pair, by subtracting the % germination of the decreaser species from the % germination of the increaser species. As such, a positive DGP indicates that, once that the effect of infected and dead seeds is removed, the increaser species germinates more than its decreaser counterpart, and vice versa. We repeated this process for T50 (DT50) and the % of infected seeds (DIP). Although the original percentages of germination and infection did not accommodate to a normal distribution, the differences did so, thus enabling us to use models with Gaussian errors. We developed a linear mixed effect model (Bates, 2005) for each of the studied response variables (DGP. DT50 and DIP), setting the effect of the pair of species as a grouping factor with random effects. In the cases of DGP and DT50, we used dung addition, fungicide addition, and their interaction as the fixed effects explanatory variables. However, because we did not find any fungal infection in the treatments that included fungicide, we excluded these treatments (CF and EF) from the analyses of fungal infection, using only dung addition as explanatory variable.

Finally, and to further explore if dung affects infection rate for increasers and decreasers separately, we performed separate mixed models with binomial errors for each group of species. In these case, the percentage of infected seeds in each dish was

the response variable, with dung addition as the fixed effects variable and species as a random effect. All analyses were conducted using the R v3.1.1 statistical package (R Development Core Team. 2014).

3. RESULTS

The model for DGP revealed that the 'pair' factor accounted for 70.6 % of the variability in DGP. Interestingly, decreaser species generally displayed higher germination percentages than increasers (negative DGP values). These differences were not affected by any of the applied treatments (Table 1). In addition, the differences in the speed of the germination (DT50) were not significantly affected by any of the studied factors (Table 1; Fig 1B). These results indicate that the treatments did not affect the germination process (neither the germination success nor its speed) of living seeds. In this sense, the lack of any significant effect of fungicide addition (i.e. between the C and CF treatments) confirms that fungicide does not affect the germination process of the studied species. The 'pair' random factor accounted for 31.1% of the variability in DT50.

None of the seeds recognised as infected germinated. Again, the 'pair' factor accounted for a great proportion of the variability in DIP (64.4%). Among the treatments that did not include fungicide addition, DIP became strongly negative in the E treatment (Fig. 1C), associated to a substantial increase (more than two times higher than in the Control) in the rate of infection of decreaser species (Fig. 1C). The separated models for each grazing response on infection percentage revealed that dung addition did not affected this parameter in increaser species (χ^2=2.11, p=0.146), whereas it significantly increased the infection percentage of decreaser species (χ^2=19.86; p<0.001). Interestingly, the effect of dung leachates on DIP was greater for species in the *Fabaceae* family (Appendix 1-Table 4) than for the rest of the families, which might suggest that this family alone determines this result. Consequently, we repeated the analysis with the other families, finding that although the effect of dung leachates on DIP was reduced, it remained highly significant after excluding the *Fabaceae* family (Dung effect: $F_{1,54}$=14.24; p<0.001).

4. DISCUSSION

The results of this study show that there is a species-specific effect of dung leachates on the germinative success of the seeds of Mediterranean annual plants: the

addition of dung leachates reduced the proportion of seeds of decreaser species that were finally able to germinate, and did not affect that of increasers. Decreaser species were more prone to be affected by pathogens in control conditions than increasers, and the addition of dung leachates further increased the differences in susceptibility. In fact, the lack of differences in germination percentages and speed of the remaining seeds (those that were viable after accounting for fungal mortality) indicates that increased mortality of decreasers under the influence of dung leachates entirely explains the different effects of dung leachates on the germination success between species with different grazing response. This was further confirmed by the addition of fungicide to dung leachates (EF treatment), which led to a total disappearance of the deleterious effect of dung leachate on the infection rates of decreaser species. Our results suggest that the different germination responses to dung leachates displayed by increaser and decreaser species can be ultimately caused by differences in their sensitivity to the activity of fungal pathogens.

These results entail two interesting implications. First, they add dung leachates to the list of factors that can affect the effect of fungal pathogens on seeds. Second, and in agreement with our initial hypothesis, they provide support for the role of pathogenic fungi as determinants of the different effects of dung leachate on the germination of species with different grazing response. Previous studies have described a higher fungal activity under increased moisture conditions (Mordecai, 2012; Schafer and Kotanen, 2003), but this is the first time, to our knowledge, that livestock activity is related with these changes. However, for fungal effects to be one of the causes behind the different grazing responses of increaser and decreaser species, the relationship between environmental conditions and fungal attack is not sufficient. Besides this, differences in the effect of fungal pathogens on seed mortality must be species-specific, with some species being favoured (increasers) in relation to others (decreasers) by the change in the conditions (grazing). Other authors have reported similar mechanisms, like the better capacity of the seeds of species of wet grasslands to resist the anoxic conditions associated to a groundwater level close to the soil surface than those of species from dry grasslands (Bekker et al., 1998), or the species-specific effect of soil moisture in the susceptibility to fungal infections reported by Schafer and Kotanen (2003). Moving the focus specifically to the effect of grazing activities, Dobarro et al. (2010) showed that increasers germinate less than

decreasers when the red/far red ratio of the incoming light is similar to that observed in ungrazed conditions. Our results show that increaser species are favoured in comparison to decreaser species under the influence of dung leachates, and that this advantage is due to their higher resistance to infections by pathogenic fungi.

Our results raise a set of new potential questions that would require the performance of new experiments to be answered. First, the seeds of increaser species were not affected by pathogens (Fig. 1), as revealed by the low infection rates found for the treatments without fungicides. This suggests that the seeds of these species could have antifungal compounds (Orrock and Damschen, 2005), resulting in a relative advantage for increasers compared with decreaser species, which were more susceptible to fungal infections (Mitschunas et al., 2006). In addition, it is unclear whether other components of the soil biota, such as mycorrhizal fungi or fungivorous invertebrates (Mitschunas et al., 2006) might affect the balance found in this experiment. It may be also interesting to ascertain what is the source of the fungal pathogens observed in this study, as well as its taxonomic classification. Faeces of mammals are known to contain the spores of certain fungi, therefore acting as dispersal agents (Nuñez et al., 2013; Wood et al., 2015). Another possibility is that the spores were attached to the seeds themselves, and the input of nutrients associated to dung leachates would have acted as a trigger of the fungal pathogens. An experiment in which seeds were sterilized before the addition of dung leachates (e.g. Schafer and Kotanen 2004) would help to answer this question.

In spite of the great relevance of livestock grazing as a factor influencing the species composition of grasslands at a global level, the effects of the different livestock activities are still poorly understood. Carmona et al. (2013) shown that leachate addition significantly reduced the germination percentage and speed of decreaser species, whereas increaser species displayed a greater capacity to tolerate dung leachate environments. These different responses can in turn result in differences in the colonising abilities of both groups of species, especially considering the profusion of dung pats in grazed areas (Bakker and Olff, 2003; Carmona et al., 2013), and its persistence in the field (Dai, 2000). Indeed, there are many other factors apart from dung deposition, which can have synergistic and species-specific effects, and is the final balance of all these grazing-related factors what determines the compositional differences between grazed and ungrazed areas. These include different

resistance to defoliation between species (Del-Val and Crawley, 2005, 2004), changes in soil fertility (Peco et al., 2006), changes in light quality (Dobarro et al., 2010) or biomass destruction by trampling (Dobarro et al., 2013). Importantly, the considerable variation among families (as revealed by the high proportion of variability explained by the 'pair' factor in all the statistical analyses) suggests that future studied trying to understand these differences should control for the effect of phylogeny.

To conclude, we want to stress that this is only a first experiment to test the role of fungal pathogens on the differential effects of dung leachates on increaser and decreaser species. A confirmation of the potential significance of this mechanism would require further experiments under field conditions, thus including a variety of factors that were not considered in this experiment. Nevertheless, our results are consistent with previous field evidence in the same region showing that several of the selected increaser species have the ability to germinate in cattle dung (Malo and Suárez, 1995a, 1995b). These species include *A. pelecinus*, *P. coronopus*, *P. annua*, *S. purpurea*, *T. glomeratum* and *V. muralis*, all of which were not negatively affected by dung leachates in the present experiment. In contrast, the decreasers *V. lathyroides* and *A. granatense* were not able to germinate in cattle dung (Malo and Suárez, 1995a, 1995b), in a way that is consistent with our results. In any case, the results presented here, along with those of Carmona et al. (2013) provide support for the interpretation that the presence of livestock, and the associated deposition of dung, modulates the activity of pathogenic fungi on the seeds of annual species. This, along with the different vulnerabilities of species to fungal attacks might be one of the causes that ultimately lead to the different specific compositions between grazed and ungrazed areas.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Appendix 1-Table 1. Summary of the results of previous studies used to determine the increaser or decreaser response to grazing of each species.

Appendix 1-Table 2. Mean of germination percentage for each species and treatment.

Appendix 1-Table 3. Mean of time to 50% of germination (T50) for each species and treatment.

Appendix 1-Table 4. Mean of percentage of infected seeds for each species and treatment.

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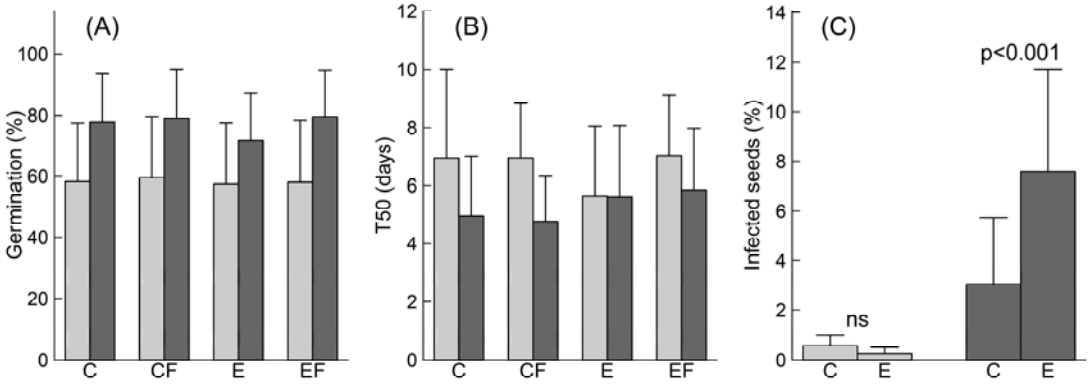
449

Table 1. Results of the mixed models analysing the effects of the different experimental factors, dung leachates addition (Dung), fungicide addition (Fungicide) and their interaction, on differences in germination percentage (DPG), T50 (DT50) and proportion of infected seeds (DIP) between increaser and decreaser species. Because no infected seeds were found on any of the treatments including fungicide, these samples were excluded from the DIP analysis. The pairs of species were used as a grouping factor with random effects. Significant results ($p < 0.05$) are in bold.

		DGP			DT50			DIP		
	Num. df	Den. df	F	p	Den. df	F	p	Den. df	F	p
Intercept	1	152	**3.96**	**0.048**	125	1.65	0.202	74	3.95	0.051
Dung	1	152	0.25	0.620	125	0.39	0.256	74	**23.87**	**<0.001**
Fungicide	1	152	0.81	0.369	125	3.35	0.071	74	~	~
Dung:Fungicide	1	152	2.55	0.112	125	0.28	0.600	74	~	~



458

459 **Figure 1.** Effect of the different treatments (control, C; fungicide, CF; dung leachates,
460 E; dung leachates and fungicide, EF; error bars represent standard error of the mean) on
461 the germination percentage (A), T50 (B) and proportion of infected seeds (C) of
462 increaser (light bars) and decreaser (dark bars) species. Because no infected seeds were
463 found on any of the treatments including fungicide, these treatments are excluded from
464 panel (C), which also displays the results of the models analysing the effect of dung
465 leachates on the infection rates of increasers and decreasers, respectively.