The effects of salt stress and arbuscular mycorrhiza on plant neighbour effects and self-thinning

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Abstract

Abiotic and biotic factors can alter the nature and strength of plant–plant interactions and therefore self-thinning (density-dependent mortality), but few studies have looked at how such factors interact. We investigated how salt stress and arbuscular mycorrhizal fungi (AMF) influence plant neighbour effects and self-thinning in experimental populations of Medicago sativa. We obtained two mycorrhizal levels by applying the fungicide benomyl (low AMF) or not (high AMF) at three salinity levels (0.05%, 0.2% and 0.5%). In experiment 1, we investigated how salinity and AMF interact to influence plant interaction intensity using a neighbour removal treatment. In experiment 2, we investigated how self-thinning dynamics vary under salinity conditions and different AMF levels at two initial plant densities (6000 and 17,500 seeds m\textsuperscript{-2}). Shoot biomass and plant density were measured 30, 60 and 90 days after sowing. Standardized major axis regression was used to estimate self-thinning parameters. In experiment 1, AMF increased competitive plant neighbour effects when there was no salinity stress, but this enhancement was not significant with increasing salinity. In experiment 2, there were effects of salinity and AMF on the self-thinning trajectory. The slope of the log (mean shoot biomass per unit area) vs. log density relationship was significantly steeper for the high AMF treatment than for the low AMF treatment without salinity, but the effect of AMF level on the self-thinning exponent was not significant under the two higher salinity levels. The effect of AMF treatments on the intercept of the self-thinning line was not significant at 0.2% salinity but was significant at 0.5% salinity, higher elevation for high AMF treatment. In self-thinning populations, AMF decreased the survival rate without salinity, but increased the survival rate at the highest salinity level. Our results support the hypothesis that salinity and AMF interact to influence plant neighbour effects and self-thinning. Under no-salinity conditions, AMF increased competition, steepened the self-thinning line and decreased survival rate, but these effects of AMF were not significant in the presence of salinity.

Zusammenfassung

Abiotische und biotische Faktoren können die Natur und Stärke der Interaktionen zwischen Pflanzen verändern und daher selbstausdünnend wirken (dichteabhängige Mortalität), aber nur wenige Untersuchungen haben darauf geschaut, wie diese Faktoren interagieren. Wir untersuchten wie Salzstress und arbuskuläre Mykorrhizapilze (AMF) den Einfluss der pflanzlichen Nachbarn und die Selbstausdünnung bei experimentellen Populationen von Medicago sativa beeinflussen. Wir erreichten zwei Grade von Mykorrhiza, indem wir bei drei Salinitäten (0.05%, 0.2% und 0.5%) das Fungizid Benomyl anwenden (geringe AMF) oder auch nicht (hohe AMF). In Experiment 1 untersuchten wir, wie Salinität und AMF bei der Beeinflussung...

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Introduction

Plant–plant interactions are among the most important factors regulating the dynamics of plant populations (Morris 2003) and the structure of plant communities (Callaway 1995). The nature and strength of plant interactions can be altered by the abiotic and biotic context (Agrawal et al. 2007). In severe environments (for example arctic, alpine, desert or salt marsh systems), facilitative plant–plant interactions seem to be common (Callaway 1995; Brooker & Callaghan 1998), whereas in more benign environments competitive interactions dominate. Therefore variation in environmental conditions may shift the relative importance of positive and negative interactions and change the net outcome of plant–plant interactions (Agrawal et al. 2007). For example, in abiotically harsh environments, facilitation may contribute more than negative interactions to individual plant performance, species distributions and community composition (e.g. Brooker & Callaghan 1998; Kikvidze et al. 2005).

Biotic factors, such as consumer pressure (Smit, Rietkerk, & Wassen 2009; Bulleri, Cristaudo, Alestra, & Benedetti-Cecchi 2011), parasitism (Grewell 2008) and symbiosis (i.e. mycorrhiza; van der Heijden & Horton 2009) can also mediate plant interactions. Mycorrhiza enhanced the competitive ability of C₄ over C₃ plants (Tang, Xu, & Hu 2009). Soil pathogenic fungi affected the co-existence of two tall grass prairie species (Holah & Alexander 1999). Herbivory decreased intraspecific competition but increased interspecific competition in two aquatic species (Center et al. 2005). Abiotic and biotic factors interact in natural ecosystems. Mycorrhizal mediation of plant–plant interactions has been shown to be dependent on physical environmental factors such as drought stress (Zhang, Xu, Tang, & Chen 2011), CO₂ elevation (Chen et al. 2007) and nitrogen enrichment (Johnson, Rowland, Corkidi, Egerton-Warburton, & Allen 2003). We need to investigate more of these interactions if we are to understand plant–plant interactions in the field.

Self-thinning (density-dependent mortality) is an important process in plant population dynamics, which is driven by competition among individual plants. The self-thinning process is usually described in terms of the trajectory of changes in log (mean individual mass [MI]) or log (total mass [BI]) of surviving plants vs. log (number of survivors per unit area [N]; Yoda, Kira, Ogawa, & Hozumi 1963; Morris 1999, 2002). Both abiotic and biotic factors can alter this trajectory. Fertility levels or water stress is associated with changes in the slope of biomass–log density relationships (Morris & Myerscough 1991; Morris 2002, 2003; Deng et al. 2006). Arbuscular mycorrhizal fungi (AMF) increased shoot biomass allocation, enhanced aboveground competition, and made the self-thinning trajectory of Medicago sativa steeper (Zhang, Zhang, Weiner, Tang, & Chen 2011). The species of arbuscular mycorrhizal fungi can also affect the speed of self-thinning by influencing plant interactions (Zhang, Tang, & Chen 2011). Few studies have investigated interactions between abiotic and biotic factors that influence self-thinning dynamics (Le Roux & McGeoch 2010). Here we present a study about the effects of salinity stress and AMF on plant neighbour effects and self-thinning.

AMF form symbiotic associations with roots of most terrestrial plants, affecting nutrient uptake, plant–plant interactions (Moora & Zobel 1996), population dynamics (Zhang, Zhang, et al. 2011) and plant community structure (van der Heijden, Wiemken, & Sanders 2003; Vogelsang, Reynolds, & Bever 2006). Salinity is a common environmental stress that can affect interactions among plants (Callaway 1995). Along a gradient of increasing salinity, net plant neighbour effects changed from competition to facilitation (Bertness...
& Shumway, 1993; Bertness & Callaway 1994). Soil salinity can interact with consumer pressure to alter plant-plant interactions (Crain 2008). Salinity can interact with AMF to influence physiological processes and individual performance (Sharifi, Ghorbanli, & Ebrahimzadeh 2007; Daei, Ardekani, Rejali, Teimuri, & Miransari 2009). Potential interactions between salinity and AMF on plant-plant interactions have not been investigated.

We hypothesize that the effects of salinity stress, reduced growth and increased positive neighbour effects, may be reversed by AMF which enhance plant growth, competition and therefore self-thinning. We conducted two experiments with M. sativa L. to test this hypothesis. To establish a low-AMF treatment, we used the fungicide benomyl to reduce the AMF level. It has been demonstrated that the primary effects of benomyl on M. sativa grown in the same soil as used in this study are due to the suppressed AMF colonization (Zhang, Zhang, et al. 2011). Benomyl application has been widely used to reduce AMF in scientific research because it appears to have little or no direct effects on the non-mycorrhizal plant and bacterial community (Daleo et al. 2008).

Materials and methods

We conducted two experiments in this study. The first examines the response of target plants to neighbours, and the second addresses density-dependent mortality under various salinity levels and AMF statuses. The soil and seeds of M. sativa L. are described in Appendix A: SM1.

Experiment 1: neighbour effects

The experiment was a three-factor design with (i) neighbour treatment (with or without neighbours), (ii) two AMF levels and (iii) three salinity levels. The salinity levels were 0.05% (original soil, no salinity stress), 0.2% (medium salinity stress) and 0.5% (high salinity stress). The original soil salt content was considered as “no salinity stress” treatment, the other two salinity levels were created by adding sea salt to the original soil (details provided in Appendix A: SM2). Before and after the experiments, electrical conductivity was measured (see Appendix A: Table 1). The AMF levels were “low AMF” (obtained by applying fungicide), and “high AMF” (no fungicide) (see Appendix A: SM2). There were four replicates for each treatment.

Twenty seeds were sown in each container (47.5 cm length × 34.5 cm width × 15.4 cm height, filled with 20 kg of soil). One week after germination, seedlings were thinned manually three times to obtain the “without neighbours” treatment (solo plants) and the “with neighbour treatment” (target +10 neighbours, see Appendix A: SM3). All microcosms were arranged in a completely randomized design under greenhouse conditions with an average air temperature of 18–30 °C during the course of the experiment from April to August. Plants were watered daily to keep soil moisture at 70–90% of water-holding capacity. No additional nutrients were added during the experiment.

Target plants were sampled 120 days after sowing. Shoot biomass was measured after drying at 65 °C for 48 h. Root systems were collected and cleaned for AM colonization detection by the gridline intersection method (Giovannetti & Mosse 1980). The presence or absence of mycorrhizal infection (arbuscules, vesicles, coils or hyphae) was then evaluated using a compound microscope at 200× magnification. The AMF colonization level was calculated as: AMF colonization (%) = (no. of intersections colonized (hyphae, arbuscules, vesicles and hyphal coils)/total no. of intersections examined) × 100.

The relative interaction index (RII) was calculated as: (Bn − Bc)/(Bn + Bc), Where Bn and Bc are the shoot biomass of target plants in the “with neighbours” and “without neighbours” treatment, respectively. Positive values indicate that the overall effect of neighbours on the target plant is positive (facilitation), while negative values indicate that the net effect of neighbours is negative (competition).

Three-way analysis of variance (ANOVA) was performed by SPSS (V.10.0) on AMF colonization and biomass of target plants. The AMF level, neighbour treatment and salinity level were considered as the three factors. Two-way analysis of variance (ANOVA) was performed on RII with the AMF and salinity level as the two factors. Normality tests were performed before ANOVA. The least significant difference (LSD) at the 5% confidence level was used for comparisons.

Experiment 2: self-thinning

The experiment was a three factor design with (i) three salinity levels, (ii) two AMF levels, and (iii) two initial plant densities (6000 and 17,500 seeds m−2). Three salinity levels and the AMF treatments were the same as experiment 1. There were four replicates for each treatment.

Containers were the same as experiment 1. M. sativa seeds were surface sterilized with 1% NaClO and distilled water, mixed with sand and sown with a sieve to achieve a random spatial pattern. Containers were randomly arranged in a greenhouse condition that was the same as experiment 1 from April to August.

Sampling was carried out at 30, 60 and 90 days after sowing, with 25, 36 and 100 cm2 square plots respectively, as described by Shumway and Koide (1995). Detailed sampling method was described in Appendix A: SM4. Shoots and roots were oven dried at 65 °C for 48 h and weighed. Mean biomass for shoots and roots was calculated by dividing the total biomass by the number of individuals. AMF colonization rate of root was quantified using the grid-method as in experiment 1.

The self-thinning intercepts and the slopes were estimated by the standardized major axis (SMA; SMATR Version 2.0; Warton, Wright, Falster, & Westoby 2006) regression on log-transformed data of density and aboveground biomass of surviving plants per unit area (Morris 2002; Zhang, Zhang,
et al. 2011). We compared slopes of biomass–density relationship between the two AMF levels by using SMATR (Version 2.0). For testing difference in elevation between AMF treatments we used WALD statistic model in SMATR. Pre-thinning vs. thinning data points were separated according to the criteria of Morris (1996), and only the data from self-thinning populations were included in the analyses (Morris 2002). There are 16 data points in each group in the analysis.

The survival rate was calculated as final plant density/initial plant density × 100%, where initial plant density is calculated from emergence (Zhang, Tang, et al. 2011) and final plant density is the density of surviving plants at the last sampling.

SPSS (V.10.0) was used to perform a three-way analysis of variance (ANOVA) on AMF colonization and survival rate with AMF level, plant density and salinity as factors. Normality tests and a homogeneity test were performed before ANOVA. The least significant difference (l.s.d.) at the 5% confident level was used for comparisons.

**Results**

**Experiment 1**

**Mycorrhizal colonization**

Benomyl application and high salinity levels significantly decreased AMF colonization, but neighbour treatment did not (Fig. 1, Appendix A: Table 2). There were no significant interaction effects between salinity and neighbours (see Appendix A: Table 2) or between benomyl application and neighbours (see Appendix A: Table 2) on AMF colonization, but salt stress and benomyl application interactively affected on fungal colonization (see Appendix A: Table 2).

**Biomass of target plants**

There were significant interactions among salinity, benomyl application and neighbour treatment in their effects on biomass of target plants (see Appendix A: Table 3). All 2-way and the 3-way interaction among these variables were highly significant. Salinity, neighbour treatments and their interaction exerted the strongest effects on biomass of target plant (Fig. 2, Appendix A: Table 3).

**Neighbour effect**

The relative interaction index (RII) values were negative in all treatments, which means that neighbour effects were competitive (Fig. 3). The RII was less negative with increasing salinity, indicating that the competition intensity decreased in the high salt stress treatment. Mycorrhizal influence on neighbour effect depended on the salinity treatment (Fig. 3,
Appendix A: Table 4). Under low salt stress treatment, AMF enhanced competition intensity significantly. However, under medium and high salt stress treatment, the influences of AMF were not significant (Fig. 3, Appendix A: Table 4).

Experiment 2

Colonization rate

AMF colonization was not significantly affected by the interaction of salinity, benomyl application and plant density (see Appendix A: Table 5), but was significantly influenced by the interactions of salinity level and plant density (see Appendix A: Table 5), salinity level and benomyl application (see Appendix A: Table 5), benomyl application and plant density (see Appendix A: Table 5). Benomyl application and salinity stress significantly decreased AMF colonization (Fig. 4, Appendix A: Table 5). Under the two higher salinity levels, AMF colonization increased with increasing plant density (Fig. 4, Appendix A: Table 5).

Biomass–density relationship

In the no salinity treatment, the slope of the log biomass–log density relationship was steeper in the high AMF treatment ($p = 0.0026$; Fig. 5A and Table 1). The slope of log biomass–log density relationship was not significantly altered at 0.2% soil salinity ($p = 0.4955$; Fig. 5B and Table 1) or the 0.5% soil salinity level (for slope: $p = 0.2848$; Fig. 5C and Table 1). Only the population in the control salinity level showed an increase in log shoot biomass as self-thinning progressed (Fig. 5A and Table 1). The biomass decreased as density declined under the two high salinity levels with positive slopes (Fig. 5B, C and Table 1).

Testing for shifts in elevation of the self-thinning trajectory between groups using the Wald statistic showed a significant elevation shift between AMF treatments at the 0.5% soil salinity ($p = 0.0406$, higher elevation for the high AMF treatment), but no significantly different result at the 0.2% soil salinity ($p = 0.439$).

Survival rate

The survival rate of *M. sativa* decreased with increasing salinity and initial plant density treatments (Fig. 6, Appendix A: Table 6). Benomyl increased the survival rate during self-thinning under no salinity, whereas it decreased survival rate at the highest salinity level (Fig. 6, Appendix A: Table 6). The survival rate was not significantly affected by the interaction of salinity, density and benomyl application (see Appendix A: Table 6), and the interaction of benomyl application and seedling density, but was significantly affected by the interactions of initial density and salinity treatment (see Appendix A: Table 6), and by benomyl application and salinity (see Appendix A: Table 6).

Discussion

Both experiments showed that the application of benomyl suppressed AMF colonization (Figs. 1 and 4). As in our
Table 1. Regression parameter estimates (standardized major axis regression) of log (shoot biomass) on log density in populations of Medicago sativa L. undergoing self-thinning under “high AMF” and “low AMF” treatments along a salinity gradient. “Low AMF” was obtained with fungicide application and “high AMF” without fungicide.

<table>
<thead>
<tr>
<th>AMF treatment</th>
<th>Salinity treatment</th>
<th>n</th>
<th>Intercept</th>
<th>Slope</th>
<th>95% CI of slope</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>“High AMF”</td>
<td>No stress</td>
<td>16</td>
<td>5.11985</td>
<td>−0.6857</td>
<td>(−0.9021, −0.5213)</td>
<td>0.765</td>
</tr>
<tr>
<td>“Low AMF”</td>
<td></td>
<td>16</td>
<td>4.24367</td>
<td>−0.416</td>
<td>(−0.7114, −0.2432)</td>
<td>0.036</td>
</tr>
<tr>
<td>“High AMF”</td>
<td>Medium stress</td>
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<td>0.46727</td>
<td>0.5762</td>
<td>(0.3357, 0.9893)</td>
<td>0.021</td>
</tr>
<tr>
<td>“Low AMF”</td>
<td></td>
<td>16</td>
<td>−0.06765</td>
<td>0.7067</td>
<td>(0.4170, 1.1975)</td>
<td>0.072</td>
</tr>
<tr>
<td>“High AMF”</td>
<td>High stress</td>
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<td>−0.51736</td>
<td>0.8212</td>
<td>(0.6103, 1.105)</td>
<td>0.724</td>
</tr>
<tr>
<td>“Low AMF”</td>
<td></td>
<td>16</td>
<td>−0.54345</td>
<td>0.8032</td>
<td>(0.6543, 0.986)</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Fig. 6. Survival rates of Medicago sativa L. populations grown under different treatments (experiment 2). “LM”, low AMF (fungicide application) and “HM”, high AMF (without fungicide). “LD”, low plant density (6000 seeds m$^{-2}$) and “HD”, high plant density (17,500 seeds m$^{-2}$).

previous studies (Zhang, Xu, et al. 2011; Zhang, Zhang, et al. 2011), suppression of AMF colonization decreased growth of M. sativa, supporting the use of fungicide as a way to decrease AMF colonization.

Our results support the stress gradient hypothesis, which holds that plant interactions become less negative along a gradient of increasing environmental stress (Maestre, Bautista, & Cortina 2003; Lortie & Callaway 2006). In experiment 1, shoot biomass of target plants decreased as salinity stress increased regardless of the presence of neighbours or AMF levels, but the magnitude of this decrease was lower with neighbours regardless of AMF levels. Plant neighbour effects became less negative as salinity stress increased regardless of AMF levels, although they never became positive in the present study.

Results from experiment 1 support our hypothesis that salinity stress and AMF interact to affect plant neighbour effects. Although high AMF enhanced biomass of solo plants at all salinity levels, a strong negative neighbour effect due to high AMF only occurred without salinity. The simplest explanation for this is that AMF increases plant growth rate and therefore the effects of competition, but it cannot do this in the presence of salinity. Mycorrhizae usually increase aboveground competition for light among plants by enhancing plant growth (Smith & Read 2008). High AMF can also increase allocation of biomass to shoots at a given biomass, resulting in increased aboveground competition (Zhang, Zhang, et al. 2011). An alternative explanation is that mycorrhizal fungal hyphae can form a belowground network, connect neighbouring plants and share soil nutrient among the connecting individuals, and this can decrease negative and increase positive effects from neighbouring plants (van der Heijden & Horton 2009).

Self-thinning dynamics can be affected by abiotic factors, such as fertility, water availability (Morris 2002, 2003; Deng et al. 2006; Chu et al. 2010) and biotic factors such as mycorrhizal fungi and consumers (Crain 2008; Bulleri et al. 2011; Zhang, Xu, et al. 2011; Zhang, Zhang, et al. 2011). Mycorrhizal symbiosis is an important biotic factor that can increase shoot branching, canopy radius, aboveground biomass, and allometric growth (Shumway & Koide 1995; Zhang, Zhang, et al. 2011). The mycorrhiza-induced enhancements of individual plant growth and canopy occupation increase aboveground competition among individual plants within a population, and can result in a steeper self-thinning line (Zhang, Zhang, et al. 2011). Our results from experiment 2 demonstrate that the self-thinning trajectory was also influenced by an interaction between salinity and mycorrhiza. From the controls to the highest salinity level, there is a progression in slope and relative position of the self-thinning lines for the two AMF treatments. In the absence of salinity, biomass per unit area increased with decreasing plant density, and the position of the self-thinning line of high AMF was below that of low AMF (Fig. 5A). At the 0.2% treatment the slope and relative position of the two self-thinning lines shifted so that the line for the high AMF treatment lay above that for the low AMF treatment, but the effect was not significant (Fig. 5B). At 0.5% salinity, the shift in slope and relative position of self-thinning lines became more obvious: biomass per unit area decreased with decreasing plant density under both AMF levels, and the position of the self-thinning line for high AMF was significantly above the one for low AMF. Mycorrhizal mediation of plant neighbour effects point us towards new hypotheses to explain the dynamics of plant populations. In our study, a steeper plant self-thinning line with high AMF and no salinity stress...
could be due to mycorrhizal enhancement of growth and therefore negative neighbour effects, because the strength of competition at a given density increases with plant growth. Conversely, the positive slopes of the self-thinning lines under the two levels of salinity stress may result because mycorrhiza did not increase the negative neighbour effect. Moreover, root colonization in the high AMF treatment increased with plant density at the higher salinity treatments, but not in the no salinity control. These results support the hypothesis that the plant–AMF relationship changes along a salinity gradient, and that AMF can play a positive role and reduce the intensity of competition in self-thinning stands.

The survival rate in self-thinning populations also conveys information about plant population dynamics. Experiment 2 indicates that AMF and salinity also affect plant survival rate by altering neighbour effects. In the no salinity control, the AMF increased the growth of plants, leading to more mortality per unit time. The effect of increased growth on self-thinning has been shown in previous work on soil fertility levels (Morris 1999). This phenomenon can be explained by the mediation of mycorrhiza on plant competition. In a self-thinning population, mortality originates from intraspecific competition for limiting resources especially light (Westoby and Howell 1981; Lonsdale and Watkinson 1982). As argued above, at a given density, the larger the plants are, the more intense is competition for limiting resources (Morris 2003). In our experiment 1, mycorrhiza increased plant growth and enhanced negative neighbour effects in the absence of salinity, resulting in earlier and higher mortality. At the higher salinity levels, although mycorrhiza increased shoot biomass somewhat, they did not increase negative neighbour effects, resulting in a higher survival rate.

Mycorrhizal effects on plant mortality under various environmental stresses could have long-term consequences for plant population dynamics. In our experiments, mycorrhizae promote self-thinning in the absence of salinity. During self-thinning, it is the smallest individuals that die first because they tend to suffer more from competition (Weiner 1986; Weiner et al. 2001; Weiner and Damgaard 2006; Zhang, Tang, et al. 2011). This process would lead to a plant population with fewer large individuals. Some genes that may be crucial for surviving under stressed conditions could be lost during this self-thinning process. Under salinity stress, however, mycorrhiza help to maintain a higher survival rate, suggesting that mycorrhiza may be beneficial for the maintenance of size and gene diversity of plant population under stressed conditions.

Conclusion

In the absence of salinity, AMF enhanced competitive neighbour effects, steepened the self-thinning line and decreased survival rate. These mycorrhizal effects were reduced or reversed with increasing salinity. At the higher salinity levels there were no significant effects of mycorrhiza on plant–plant interactions or the self-thinning trajectory, but mycorrhiza increased plant survival.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.baae.2012.09.009.

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