A nurse plant benefits from facilitative interactions through mycorrhizae

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ABSTRACT

- Plant facilitation promotes coexistence by maintaining differences in the regeneration niche because some nurse species recruit under arid conditions, whereas facilitated species recruit under more mesic conditions. In one Mexican community, 95% of species recruit through facilitation; *Mimosa luisana* being a keystone nurse for many of them. *M. luisana* individuals manifest greater fitness when growing in association with their facilitated plants than when growing in isolation. This observation suggests that nurses also benefit from their facilitated plants, a benefit thought to be mediated by mycorrhizal fungi.

- Under field conditions, we experimentally tested whether mycorrhizal fungi mediate the increased fitness that *M. luisana* experiences when growing in association with its facilitated plants. We applied fungicide to the soil for nurse plants growing alone and growing in association with their facilitated plants in order to reduce the mycorrhizal colonisation of roots. We then assessed the quantity and quality of seed production of *M. luisana* in four treatments (isolated-control, isolated-fungicide, associated-control and associated-fungicide).

- Fungicide application reduced the percentage root length colonised by mycorrhizae and reduced fitness of *M. luisana* when growing in association with their facilitated plants but not when growing in isolation. This reduction was reflected in the total number of seeds, number of seeds per pod, seed mass and seed viability.

- These results suggest that nurses benefit from the presence of their facilitated plants through links established by mycorrhizae, indicating that both plants and belowground mutualistic communities are all part of one system, coexisting by means of intrinsically linked interactions.

INTRODUCTION

The recognition that aboveground and belowground communities form major components of ecosystems interacting in a permanent feedback has been a major step towards a comprehensive understanding of species coexistence and vegetation dynamics (Wardle et al. 2004; Sanon et al. 2009; Van der Putten 2009; Bardgett & Wardle 2010; Fukami & Nakajima 2013; Van der Putten et al. 2013). For example, plant diversity can be explained in terms of a process of soil community feedback, through which both the density and composition of microbes alter in response to the composition of the plant community and, subsequently, the change in microbial composition modifies performance of the plants (Bever et al. 2010).

Plant species coexistence in stressful environments is mediated by facilitative interactions among plants that are tightly linked to the diversity of belowground microbial communities (Montesinos-Navarro et al. 2012a, 2015; Rodríguez-Echeverría et al. 2015). Facilitative interactions result in strong spatial associations between a variable number of species and a nurse species. A high proportion of these early associations is maintained through time until facilitated plants reach the adult stage, forming discrete vegetation patches surrounded by open space (Valiente-Banuet & Verdú 2007). These vegetation patches are ubiquitous in many ecosystems (Hacker & Gaines 1997; Eccles et al. 1999; Castillo et al. 2010) and constitute the context in which new individuals belonging to different species arrive via seed dispersal. Species coexistence in vegetation patches will ultimately be determined by the relative performance of the newcomers and the nurse species. In this process, the role of mycorrhizal fungi in interconnecting plants providing pathways for the transference of nutrients has become of central interest to understand benefits among the species involved in the plant facilitation process (Van der Heijden & Horton 2009; Van der Putten 2009; Montesinos-Navarro et al. 2012b).

Most studies have focused on the mycorrhizal network effects on the facilitated seedlings (Nara & Hogotsu 2004; Dickie et al. 2006), but nurses are also plugged in to the network and can receive either positive or negative effects. Previous studies show the mutual benefit in facilitative interactions. For example, Pugnaire et al. (1996) showed that *Marrubium vulgare* individuals increase in specific leaf area, leaf mass, shoot mass, N content and produce more flowers when grown
in association with the shrub *Retama sphaerocarpa*. Similarly, biomass of 1-year-old cladodes, total biomass and total N content of 3-year-old branches of *R. sphaerocarpa* shrubs were all higher when *M. vulgare* individuals were growing beneath them.

Similarly, the nurse *Mimosa luisana* produces more and better seeds when growing associated to its facilitated species in phylogenetically diverse neighbourhoods (Sortibrán et al. 2014), while the facilitated species receive N from the nurse (Montesinos-Navarro et al. 2016, 2017). Positive effects of facilitated plants on nurses would explain the maintenance of facilitative interactions until adult stages in vegetation patches, and such effects could be mediated by mycorrhizal fungi (Verdú et al. 2010). Mycorrhizal fungi can transport water, nutrients and chemical defences from facilitated plants directly to their nurses through common mycorrhizal networks (Newnan 1988; Selosse et al. 2006; Simard et al. 2012; Gorzelak et al. 2015) or indirectly through hydraulic lift or hyphal exudates (Querejeta et al. 2003; Finlay 2008; Zhang et al. 2016). Direct transfer of nutrients and water between plant roots through mycorrhizal networks has been extensively documented (Giovannetti et al. 2004; Egerton-Warburton et al. 2007; Mikkelsen et al. 2008; Simard et al. 2012; Montesinos-Navarro et al. 2016), but its ecological role on the outcome of plant–plant interactions and community dynamics is still debated (Van der Heijden & Horton 2009; Bever et al. 2010). Indirect transfer may also occur as mycorrhizal fungi can move water from roots to soil, enhancing the magnitude of hydraulic lift and making water available for other plants (Querejeta et al. 2003; Prieto et al. 2012). Similarly, mycorrhizal exudates from facilitated plants may alter bacterial communities and accelerate mineralisation rates, increasing the availability of nutrients in soil (Zhang et al. 2016). All of these mechanisms may explain how nurses can benefit from their facilitated plants through mycorrhizal fungi. However, there is contradictory evidence about the costs of plant facilitation for nurse species during the ontogeny of the interacting plants. In some cases, plant facilitation shifts to competition and nurse species fitness is reduced (Schöb et al. 2014a,b,c), which contrasts with other situations where facilitation shifts to mutualism and nurse fitness increases (Sortibrán et al. 2014). Aboveground factors (e.g. light, pollinators, herbivores) have been shown to drive the positive and negative feedback effects of beneficiary species on their nurses (Wardle et al. 2004; Wolfe et al. 2005; Van der Putten 2009), but belowground drivers, such as plant–soil positive and negative feedbacks may also be at work (Bever et al. 2010). Indeed, the response of plants to mycorrhizal fungi ranges from mutualism to parasitism (Hoeksema et al. 2010), and therefore both positive and negative effects of mycorrhizae may be context-dependent, depending on plant and fungal species identity as well as on soil nutrient availability (Van der Heijden & Horton 2009).

Here, we explore the role of arbuscular mycorrhizal fungi (AMF) involving facilitative interactions between a keystone nurse, *M. luisana* and its facilitated plant species in a Mexican desert community governed by facilitation (Valiente-Banuet & Verdú 2007; Verdú et al. 2010; Verdú & Valiente-Banuet 2011). In this community, *M. luisana* is the only species able to recruit on open ground and therefore it acts as the primary nurse for most of the species (Verdú & Valiente-Banuet 2008). Individuals of *M. luisana* growing in isolation can also be found but in smaller numbers than the individuals growing in association. Interestingly, *M. luisana* individuals coexisting with their facilitated species have been shown to have higher fitness than isolated individuals (Sortibrán et al. 2014), although it remains unclear whether these benefits are mediated by AMF.

*Mimosa luisana* is the generalist species with the largest number of partners in the plant–AMF community network (Montesinos-Navarro et al. 2012b). Likewise, there is evidence that AMF promote N transfer from *M. luisana* to its facilitated species (Montesinos-Navarro et al. 2016), thus making it possible for facilitated species to reciprocate their benefits with the nurse through mycorrhizal fungi during the ontogeny of the interacting plants. We experimentally applied a fungicide to reduce AMF abundance in *M. luisana* nurses growing both in isolation and associated with their facilitated plants and evaluated the effect on its different fitness components (reproductive success). Based on previously explained observations, we hypothesised that a reduction in mycorrhizal fungi abundance will produce a decrease in fitness among nurses when growing in association with their facilitated plants.

**MATERIAL AND METHODS**

**Study area and species**

This study was conducted in the semiarid Valley of Zapotitlán (18°20′N, 97°28′W), a local basin in the Tehuacán–Cuicatlán Valley of Puebla state, Mexico. This region owes its aridity to the rain shadow produced by the Eastern Sierra Madre (Valiente-Banuet et al. 2000). It has an annual average rainfall of 380 mm, most of which falls during the summer months, and an annual mean temperature of 21 °C, with rare frosts (García 1988). The study site is a xeric shrubland located within the Botanical Garden ‘Helia Bravo Hollis’, a natural unmodified protected area approximately 30 km south of Tehuacán city. This vegetation is dominated by the columnar cactus *Neobuxbaumia tetetzo* and shrub species such as *Mimosa luisana*, *Masagnia parviflora*, *Ipomoea arborescens*, *Aeschynomene compacta*, *Caesalpinia melanadenia*, *Calliandra eriophylla*, *Zapoteca formosa*, *Senna wislizenii*, *Agave marmorata*, *A. macrocantha* and *Jatropha neopauciflora*, among other taxa (Valiente-Banuet et al. 2000). All of these species, with the exception of *M. luisana*, recruit through facilitation. *M. luisana* is able to facilitate 48 out of 56 species recorded for this community (Verdú & Valiente-Banuet 2008), forming discrete vegetation multispecies patches of up to 12 species and 42 individuals. The area of the vegetation patches ranges from 1 to 10 m², and all facilitated individuals are located beneath the vertical projection of the *M. luisana* canopy. Certain isolated *M. luisana* individuals can also be found separated from other vegetation patches by 1.5–2.0 m (see Figure S1).
0.80-m tall. Pink flowers, which grow in spike inflorescences, are bisexual and are produced during June, just before the beginning of the rainy season (May–August). Fructification occurs from August to October. The fruit consists of one carpel pod, with each pod containing two to eight seeds; pods are grouped into bunches of 2–10 (Grether et al. 2006).

Field experiment

Sampled vegetation patches were distributed within an area of ca. 750 m² (15 × 50 m) on an east-facing slope with an angle of inclination of between 5 to 10°. Nitrogen and phosphorus concentrations in bare soils, as well as in soil newly colonised by *M. luisana* individuals, are low, ranging from 1.4 to 1.6 g kg⁻¹ and 11.95 to 12.55 mg kg⁻¹, respectively (Sortibrán et al. 2014). Soil fertility in plots with bare soils and plots where seedlings of *M. luisana* have become established do not differ statistically, indicating that this species is able to recruit in the unfertile conditions of open ground (Sortibrán et al. 2014).

To test whether fitness benefits obtained by the nurse *M. luisana* from its facilitated plants are mediated by mycorrhizae, we applied a soil fungicide treatment in randomly selected vegetation patches where reproductive individuals of *M. luisana* (>80 cm height) lived isolated or associated with neighbours of different species. The experimental design followed a 2 × 2 factorial design, crossing fungicide treatment (fungicide versus control) with growing condition (alone versus associated). The sample sizes of each group were: (i) control-alone (n = 9 patches), (ii) fungicide-alone (n = 9), (iii) control-associated (n = 14) and (iv) fungicide-associated (n = 20). This unbalanced design corresponded to the natural abundance of isolated and associated individuals. The fungicide used was Rovral 50% (Iprodione), Bayer Mexico (Mexico City), a contact fungicide with systemic effects, very efficient for eliminating mycorrhizae without affecting soil, insects, bacteria or plant growth (Gange et al. 1990; Ganade & Brown 1997; Hernández-Dorrego & Mestre-Parés 2010).

The fungicide was applied at the rate of 2.0 g l⁻¹ water (Ganade & Brown 1997) at intervals of 3 weeks, prior to the rainy season (February–July) and six times over 2 years (2011 and 2012) to avert fungicide runoff with rain. In the control treatment, the same amount of water was added as in the fungicide treatments. Fungicide and water were administered in each patch through 6-l canisters inverted into the soil in order to slowly dispense the liquid through a small orifice. To ensure that the fungicide reached all roots in the patch, one canister was set up for each square meter, across the entire soil surface below the canopy of *M. luisana*. Root samples for AMF colonisation measurements and *M. luisana* reproduction records were conducted during September 2012, 2 months after the last fungicide application.

Fungicide efficacy on AMF colonisation

The efficacy of fungicide for reducing AMF abundance on *M. luisana* roots was evaluated by obtaining root samples from all *M. luisana* individuals by excavating three roots as far as the fine roots and cutting 20 fragments, 2 cm in length per plant. We estimated the reduction in the percentage of root colonisation of AMF following the Phillips & Hayman (1970) method, but in which root samples were stored in 50% ethanol in September 2012. In the laboratory, roots were cleared in a 10% aqueous solution of KOH (w/v) for 10 min under pressure at 120 °C, washed with 10% H₂O₂ and acidified with 1% HCl (v/v). Subsequently, roots were stained with 0.05% trypan blue (w⁻³) in lactoglycerol. The percentage of root length colonised by AMF was estimated according to the method outlined in Phillips & Hayman (1970) and Giovannetti & Mosse (1980) by placing the 20 root samples from each plant on slides, examined at 400× magnification, using an optical light microscope B201, Olympus (Lombard, IL, USA). A total of 100 fields were examined per individual. The percentages of hyphae and vesicles were scored separately by counting the fields in which those structures were present.

**Fungicide effect on reproductive success of *Mimosa luisana***

We estimated the effect of fungicide treatment on associated and isolated individuals of *M. luisana*, considering four different aspects of reproductive success: seed production, seed set, seed mass and seed viability. Seed production was assessed by counting the total number of seeds produced by each individual in the different treatments. Seed set was estimated by counting the seeds per pod in 30 pods from five individuals, randomly selected from each combination of treatments. Thirty seeds from each of those five individuals were weighed in order to estimate average seed mass, then germinated to assess seed viability. Seeds were scarified with sandpaper prior to germination tests performed in Petri dishes with wet filter paper, at 25 °C in a 12-h light/dark photoperiod. As the number of fruits may depend on the size of the plant, we estimated *M. luisana* shrub size in order to consider this as a covariate in subsequent statistical models. Plant size was estimated by assuming that *M. luisana* is an inverted cone with an elliptical base, and calculating the volume after measuring the height and two perpendicular diameters of the canopy cover.

**Statistical analysis**

We compared the effects of treatment (control versus fungicide) and the growing condition of *M. luisana* (alone versus associated) on AMF root colonisation and on different components of reproductive output of *M. luisana*, applying generalised linear models (GLM) with different error structures, depending on the nature of the dependent variable. For variables recording presence versus absence (i.e. hyphae or vesicles in roots observed in 100 fields under the light microscope) or success versus failure (i.e. seed germination), we used a binomial distribution of errors. To account for overdispersion of the model, we used the quasi-binomial family in the GLM. For calculations such as total number of seeds or number of seeds per pod, produced by *M. luisana* individuals, we applied a quasi-Poisson error distribution. Finally, for continuous variables, such as seed mass, we used Gaussian distribution of errors. The size of each plant was included in all the models as a covariate, but later removed because it did not prove significant in any case. Both normality of residuals and absence of non-linear relationships between fitted values and residuals were verified. All statistical analyses were performed with R software version 3.2.1 (R Development Core Team 2015).
RESULTS

The fungicide significantly reduced AMF hyphal colonisation in the roots of *M. luisana* individuals growing in isolation (68% reduction) or associated with other species (78% reduction; Table 1, Fig. 1A). Similarly, vesicle colonisation was significantly reduced after fungicide application of 66% and 88% in isolated and associated *M. luisana* individuals, respectively (Table 1, Fig. 1B). Following fungicide treatment, similar low levels of AMF were present in the roots of both associated and isolated *M. luisana* individuals.

The effect of fungicide on the reproductive output of *M. luisana* was dependent on growing conditions (isolated and associated) in terms of all four aspects (total number of seeds, seeds per pod, seed mass and seed viability) as indicated by significant statistical interactions (Table 1). The fungicide reduced the number of seeds in *M. luisana* individuals growing associated with their facilitated plants by 2.4 times but had no effect on those growing in isolation (Fig. 1C). Similarly, the fungicide significantly reduced the number of seeds per pod in the associated but not in the isolated *M. luisana* individuals (Fig. 1D).

Following the same trend, seed mass of *M. luisana* individuals growing in association was reduced 1.6 times by fungicide, but no effect was detected in individuals growing in isolation (Fig. 1E). Finally, fungicide reduced the seed viability of associated *M. luisana* individuals by 2.8 times, whereas it did not affect the seed viability of isolated individuals (Fig. 1F).

DISCUSSION

Our results mostly show that *M. luisana* nurses obtain fitness benefits partly from their facilitated adult plants through mycorrhizae, illustrating a mechanism by which a facilitative interaction may also be beneficial for the nurse and how plant–plant and belowground positive interactions underlie species coexistence.

Fungicide application significantly reduced seed quantity and quality of *M. luisana* when growing associated with their facilitated plants but not when growing in isolation. When fungicide was applied, AMF colonisation of *M. luisana* roots was reduced, possibly reducing the benefits received from their facilitated plants and, ultimately, reducing the number of seeds per individual, number of seeds per pod, seed mass and seed viability. As the quantity and quality of seeds are indicators of how the plant allocates resources according to nutrient availability in soil (Sinclair & Vadez 2002; Hulshof et al. 2012; Marschner 2012; DiManno & Ostertag 2016), our results indicate that fungicide had negative effects on the associated nurses.

All our results indicate that nurses benefit from their facilitated plants by means of mycorrhizal fungi. The nurse *M. luisana* is a key species in the study system because it facilitates the establishment of 86% of species in the community (Verdú & Valiente-Banuet 2008) and harbours the highest diversity of AMF (Montesinos-Navarro et al. 2012a). *M. luisana*, by facilitating other species, may increase the abundance and diversity of fungi in the rhizosphere. Supporting this idea, we have found that the mean AMF abundance in the nurse (measured as percentage of root length colonised by AMF hyphae) increases with the number of facilitated species in the patch (\( r = 0.72, P < 0.05 \)). Such increment may allow *M. luisana* to benefit directly or indirectly from the complementary functions provided by different fungi (Van der Heijden & Horton 2009; Gorzelak et al. 2015). Experimental evidence indicates that plants colonised by multiple AMF species obtain more phosphorus and grow more than those colonised by only one species (Van der Heijden et al. 1998; Johnson et al. 2004; Jansa et al. 2008). A possible mechanism through which enriched rhizospheres benefit plants is the transfer of nutrients between plants (Garg & Chandel 2010; Simard et al. 2012). Soil-derived nutrients are not only essential for the development of the AMF but are also transferred in part to the host plant itself (Leake et al. 2004; Selosse et al. 2006; Bucher 2007; Smith & Read 2008; Simard et al. 2012). Mycorrhizal fungi may also redistribute water in the soil, increasing the rate of diffusion and the absorption of nutrients (Egerton-Warburton et al. 2008).

Two lines of evidence support the existence of mutual benefits between *M. luisana* and its facilitated plants through an interchange of nutrients and water in our study system. First, by using \(^{15}\)N stable isotope, Montesinos-Navarro et al. (2016) showed that soil fungi promote *N* transfer from *M. luisana* to

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Table 1. Effects of fungicide treatment (control versus fungicide) on AMF root colonisation and reproductive output of *M. luisana* individuals growing under different conditions (isolated versus associated). Each column shows the estimate and SE of the corresponding GLM.

<table>
<thead>
<tr>
<th>Condition</th>
<th>% root colonisation</th>
<th>reproductive output</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hyphae</td>
<td>vesicles</td>
<td>seed number</td>
</tr>
<tr>
<td>Fungicide</td>
<td>0.26 ± 0.26</td>
<td>0.41 ± 0.28</td>
<td>2.23 ± 0.86*</td>
</tr>
<tr>
<td>Condition × Fungicide</td>
<td>−2.12 ± 0.29*</td>
<td>−1.31 ± 0.41*</td>
<td>0.90 ± 0.99</td>
</tr>
<tr>
<td>Fungicide</td>
<td>−0.73 ± 0.38ms</td>
<td>−1.25 ± 0.56*</td>
<td>−1.78 ± 1.00ms</td>
</tr>
</tbody>
</table>

*P* < 0.05; **P** < 0.10.
its facilitated species, while an experiment using deuterium-labelled water showed that facilitated plants may transfer water to *M. luisana* (Alicia Montesinos-Navarro *et al.*, unpublished). Similarly, it is possible that other nutrients are transferred between neighbouring plants via mycorrhizal fungi (Selosse *et al.* 2006; Mikkelsen *et al.* 2008; Van der Heijden & Horton 2009; Garg & Chandel 2010; Simard *et al.* 2012). Most of these works suggest that nutrient transfer between plants occurs through common mycorrhizal networks that physically integrate them. However, hyphal linkages in field experiments like ours are difficult to demonstrate (Perry 1995), and other mechanisms may also explain how mycorrhizal fungi mediate the fitness benefits of the nurses when living in close association with their facilitated plants. For example, our results might be explained by increased production of extra-radical mycelia in *M. luisana* individuals associated with their facilitated plants, as a result of multiple nutrients provided to fungi by facilitated plant species. More extensive extra-radical mycelia may increase water and nutrient absorption along hyphae, ultimately influencing nurse fitness. However, when *M. luisana* grows in isolation, sources of nutrients for fungi are more limited and extra-radical hyphal networks may not be as extensive as most of the soil microorganisms are carbon-starved (Berendsen *et al.* 2012). Thus, the development of the extra-radical hyphal network in isolated *M. luisana* individuals may be carbon-limited because of the high maintenance cost, which may be up to 40% of photosynthates secreted into the rhizosphere (Bais *et al.* 2006). However, once facilitated species grow associated and connected to *M. luisana* through the hyphal network, a supplementary source of nutrients may be available for fungi, allowing the formation of more extensive mycelia. This difference in the size of the extra-radical mycelia between nurses growing in association or isolation may explain why mycorrhizal fungi reduced the fitness of the former, but not that of the latter. Similarly, an extensive mycorrhizal network would enable the nurse to have greater access to the water and nutrients that their facilitated species may deliver through hydraulic lift and root exudates, processes in which mycorrhizal fungi can also participate (Prieto *et al.* 2012).

Unfortunately, using our data, it is impossible to separate the relative contributions made by these non-exclusive mechanisms. However, an unequivocal pattern of nurse benefits, apparently mediated by mycorrhizal fungi, is revealed that deserves further research.

Two potentially confounding factors to explain the increment of the quantity and quality of seed production for *M. luisana* individuals associated to its facilitated species are that nurse plants with associated species might be older or inhabit preferable microsites, where mycorrhizae are more abundant. However, our analyses show that plant size, as a proxy of age, does not correlate significantly with quantity and quality of seed production in any case. Furthermore,
microclimate variation in the study site was tested previously by Sortibrán et al. (2014) by analysing soil samples taken under *M. luisana* saplings (ca. 10-cm height) and in open areas, showing that improved soil condition under *M. luisana* adults is not a cause of initial recruitment. Consequently, we may also discard this confounding effect by showing that microhabitat amelioration is a direct effect, and not the cause of nurse establishment.

In short, contrary to traditional thinking, which suggests that competition is the main mechanism assembling ecological communities, our results show that within the context of plant facilitation, plants and belowground mutualistic communities are all part of one system, whose positive interactions ultimately determine coexistence.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1.** Study site showing three vegetation patches in which *M. luisana* grows associated with multiple species of the facilitated plants *Neobuxbaumia teteza*, *Opuntia pilifera*, *Thompsonella minutiflora*, *Mammillaria colina*, *Coryphantha pallida* and *Siphonoglossa ramosa* (white circle); growing associated to *T. minutiflora* (yellow circle); and growing in isolation (red circle). The distance between patches ranges from 1.5 to 2.5 m. Photograph by Lugui Sortibrán.

**REFERENCES**


Mutualism mediated by mycorrhizal fungi


