Universidad Nacional Autónoma de México
Doctorado en Ciencias Biomédicas
Instituto de Ecología

Mecanismos de Coexistencia y Reglas de Ensamblaje Filogenético de Comunidades Áridas

Tesis

Que para Optar por el Grado de:

Doctora en Ciencias

Presenta:

Rocío Lugui Sortibrán Martínez

Director de Tesis:

Dr. Alfonso Valiente Banuet

Instituto de Ecología

Comité Tutor:

Dra. María del Rocío Cruz Ortega

Instituto de Ecología

Dr. Pablo Vinuesa Fleischmann

Centro de Ciencias Genómicas

Ciudad Universitaria, Ciudad De México, Junio de 2018

MECANISMOS DE COEXISTENCIA Y REGLAS DE ENSAMBLAJE FILOGENÉTICO DE COMUNIDADES ÁRIDAS

ROCÍO LUGUI SORTIBRÁN MARTÍNEZ

INSTITUTO DE ECOLOGÍA, UNAM
JUNIO 2018

Esta tesis fue desarrollada en el Laboratorio de Ecología de Comunidades del Instituto de Ecología de la Universidad Nacional Autónoma de México (UNAM), bajo la dirección del Dr. Alfonso Valiente Banuet, mientras estuve en el Programa de Doctorado en Ciencias Biomédicas. Con el apoyo financiero de PAPIIT-DGAPA, UNAM (IN-202811-3; IN-213414-3), MICINN (CGL2011-29585-C02-01), CYTED (Acción 409AC0369) y la beca otorgada por CONACYT (103137). FAPESP y The São Paulo Scientific Funding Agency financiaron mi viaje y estancia en el curso SÃO PAULO ADVANCED SCIENCE SCHOOL ON ECOLOGICAL NETWORKS en Brasil, organizado por The State University of Campinas, The University of São Paulo and ABECO, The Brazilian Association for Ecological Science. ACYTED/AECID financiaron mi estancia en Cuba para el Curso-Taller Estructuras de comunidades vegetales de montaña: Patrones ecológicos y procesos históricos evolutivos.

El comité tutoral que asesoró el desarrollo de esta tesis estuvo formado por:
Dra. María del Rocío Cruz Ortega, Instituto de Ecología, UNAM
Dr. Pablo Vinuesa Fleischmann, Centro de Ciencias Genómicas, UNAM
Dr. Alfonso Valiente Banuet, Instituto de Ecología, UNAM

Miembros del jurado

Dr. Luis David Alcaraz Peraza	Presidente
Dr. Alfonso Octavio Delgado Salinas	Vocal
Dr. Erick de la Barrera Montpellier	Vocal
Dra. Ek del Val de Gortari	Vocal
Dr. Alfonso Valiente Banuet	Secretario

Figura de portada: Tomada de John Whitfield. 2007. Fungal roles in soil ecology: Underground networking. Nature 449: 136–138.

A mi familia

A mis Albertos

Agradecimientos

Quiero agradecer especialmente a mi asesor el Dr. Alfonso Valiente Banuet por su apoyo, paciencia, dedicación y enseñanza durante mi formación académica.

A la Dra. Rocío Cruz y al Dr. Pablo Vinuesa porque siempre fomentaron la discusión en los tutorales, haciendo aportaciones muy valiosas para mi investigación. Siempre con la mejor disposición.

A los miembros de mi jurado: Dra. Ek del Val, Dr. Alfonso Delgado, Dr. Erick de la Barrera, Dr. Luis Alcaraz y Dr. Alfonso Valiente por aceptar ser de mi jurado y por sus valiosas aportaciones para mejorar esta tesis.

A Carlos Silva por todo su apoyo en el trabajo de campo desde la primera salida hasta la última, siempre dispuesto a hacer el trabajo más pesado, con mucha iniciativa y solidaridad. También por su apoyo en el trabajo de laboratorio y logístico. Gracias por todo.

A Juan Pablo Castillo por su ayuda en el trabajo de campo y laboratorio. Por su solidaridad y ánimo.

A los miembros del laboratorio de Alelopatía del Instituto de Ecología, principalmente a la Dra. Rocío Cruz, Dra. Mariana del Olmo y a la Biól. Blanca Hernández por su asesoría y apoyo en el aislamiento, extracción de ADN y análisis molecular de hongos.

Al Dr. Miguel Verdú por fomentar la discusión y por sus valiosas aportaciones en los manuscritos.

A todos mis compañeros del laboratorio de Ecología de Comunidades por esos días de seminarios, su entusiasmo y amistad Andrea, Alicia, Ana Contreras, Germán, Ana Galicia, Tania, Griselda, Abril y Jazmín.

A mi compañera y amiga del doctorado la Dra. Gabriela Montes por su apoyo y entusiasmo durante todo el doctorado.

A Alberto Arizmendi por su ayuda en el trabajo de campo, sus días económicos y vacaciones fueron usados para esta investigación. Por escucharme, entenderme, involucrarse en mi (nuestra) tesis. Por animarme para seguir en los momentos más difíciles. Gracias por tú cariño y por estar aquí, ahí, acá y allá.

A mi hijo Alberto por darme alegría, amor, fuerza y otro sentido a mi vida.

A mi mamá y a mi hermana Tania por todo su apoyo incondicional en todos los ámbitos. Gracias por ser como son.

A mi tía María Elena por contar siempre con su apoyo.

A la familia Arizmendi por su solidaridad y cariño.

A todos los que influyeron de muchas maneras para concluir esta etapa de mi vida: Leo, Ane, Matt, Rocío, Alejandro, Ivette, Carlos, Fernanda, Nadia, Luza, Nancy, Ana, Rosalinda, Omar y Efrén.

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Resumen

Por décadas la teoría ecológica ha considerado solamente dos procesos: (1) Filtro Ambiental y (2) Exclusión Competitiva, como las principales reglas de ensamblaje de comunidades. Sin embargo, y en contraposición con esta visión, las interacciones ecológicas de facilitación, por las cuales hasta el 96-100% de las especies de la comunidad se establecen ayudadas por otras especies, han llegado a ser consideradas de gran importancia en la organización de las comunidades. Aún y cuando se ha mostrado que la facilitación entre especies cercanas puede cambiar a competencia a lo largo de la ontogenia de la interacción, el 57% de las asociaciones interespecíficas se mantienen hasta que los individuos facilitados llegan a la etapa adulta formando asociaciones interespecíficas. En este contexto esta tesis presenta evidencia por primera vez que las interacciones de facilitación cambian a mutualismo con respecto al tiempo para especies filogenéticamente distantes. *Mimosa luisana* Brandegeese se beneficia de crecer asociada, incrementando la cantidad y calidad de semillas, y éstas aumentan en vecindarios filogenéticamente diversos (Capítulo I). Además, se presenta evidencia hasta ahora inédita que el mutualismo entre plantas es mediado por la participación de redes de hongos micorrícicos arbusculares que conectan a *M. luisana* con sus plantas facilitadas transfiriendo nutrientes (Capítulo II). Al romper la red micorrícica con fungicida *M. luisana* produjo menor producción de semillas de menor masa y con baja viabilidad. Esta red micorrícica también participa en la trasferencia de N proveniente de la nodriza *M. luisana* a sus plantas facilitadas (Capítulo III). Las plantas facilitadas recibieron más ^{15}N en presencia de la red micorrícica que las plantas tratadas con fungicida, dando evidencia que las interacciones de facilitación persisten en el tiempo.

Los resultados indican que todas las especies interactúan con conjuntos amplios y variados de especies y que las interacciones positivas constituyen un eje conceptual necesario para lograr un entendimiento de los procesos que mantienen la biodiversidad en los ecosistemas.

Abstract

For decades, the ecological theory has considered only two processes: (1) Environmental Filter and (2) Competitive Exclusion, as the main community assembly rules. In contrast to this view, facilitative interactions, by which up to 96-100% of species of the community regenerate, have become to be considered of great importance in community organization. Even though it has been shown that facilitation between closely related species can shift to competition along the ontogeny of interaction, 57% of interspecific associations are maintained until facilitated individuals reach adulthood leading to the formation of multispecific associations. In this context, this thesis presents evidence for the first time that facilitative interactions shift to mutualism through time for phylogenetically distant species. We show that the nurse species *Mimosa luisana* Brandegeese benefits from growing associated to its facilitated species by increasing the quantity and quality of seeds (Chapter I). In addition, we present evidence that this mutualism between plants is mediated by arbuscular mycorrhizal fungi networks that connect *M. luisana* to its facilitated plants transferring nutrients (Chapter II). By experimentally breaking the mycorrhizal network with fungicide, *M. luisana* produced a lower number of seeds than a control treatment. These seeds also have low biomass and viability. This mycorrhizal network also participates in nitrogen transference from *M. luisana* to its facilitated plants (Chapter III). Thus, facilitated plants received more ^{15}N in the presence of the mycorrhizal network than those treatments in which we applied fungicide. All the results indicate that all species interact with large and varied sets of species and that positive interactions constitute a conceptual axis necessary to understand the processes that maintain biodiversity in ecosystems.

Introducción

Los procesos que determinan la membresía y la diversidad de especies en las comunidades se les ha denominado reglas de ensamblaje (Diamond 1975). Por décadas la teoría ecológica ha considerado solamente dos procesos: (1) Filtro Ambiental – cuando las condiciones ambientales físicas determinan que solamente aquellas especies adaptadas a esas condiciones formarán parte de la misma, y (2) Exclusión Competitiva –dada por la competencia entre especies por lo general con fenotipos y nichos similares (Diamond 1975; Wiens y Donoghue 2004; Webb et al. 2002) como las principales reglas de ensamblaje. Sin embargo, y en contraposición con esta visión, Valiente-Banuet et al. (2006), indican que interacciones ecológicas de facilitación, por las cuales hasta el 96-100% de las especies de la comunidad se establecen ayudadas por otras especies sin efectos negativos en las benefactoras (Valiente-Banuet y Verdú 2007), han llegado a ser consideradas de gran importancia en la organización de las comunidades de diferentes ecosistemas como en sistemas alpinos con especies "cojín" que protegen del aire helado y dan mayor humedad y retienen nutrientes para el establecimiento de otras especies. Así mismo, arbustos que protegen de la sequía a plántulas de *Pinus* y *Quercus* (Brooker et al. 2007; Anthelme et al. 2014; Cavieres et al 2014), o en bosques tropicales, donde los árboles facilitan el establecimiento de lianas y epífitas generando el sustrato para su crecimiento (Sillett y Antoine 2004; Leicht-Young et al. 2011), así como en dunas costeras en donde arbustos disminuyen el estrés salino permitiendo la regeneración de otras especies (Forey et al. 2009; Castanho y Prado 2014). Incluso en ambientes acuáticos en comunidades pantanosas o marismas ciertas plantas modifican el sustrato o la cantidad de oxígeno en el agua (Bertness y Leonard 1997), en manglares especies que disminuyen la salinidad y aumentan la cantidad de nutrientes en el sedimento (Teutli-Hernández et al. 2017). También la cobertura de algas, disminuye los cambios de temperatura y desecación en los hábitats intermareales aumentando la riqueza en las comunidades bentónicas (Scrosati 2017).

Las especies facilitadoras favorecen el establecimiento de las facilitadas de múltiples formas al disminuir el estrés proporcionando sombra y evitando la desecación (Valiente-Banuet y Ezcurra 1991; Callaway 2007), dando protección contra herbívoros (Obeso y Fernández-Calvo 2002) y modificando las propiedades biológicas, físicas y químicas del suelo ocasionadas por su asociación con microorganismos (Carrillo-García et al. 1999; Puerta-Piñero et al. 2006; Teutli-Hernandez et al. 2017). Este beneficio se ve reflejado en la sobrevivencia, crecimiento y/o reproducción de las especies facilitadas (Callaway 2007; Bronstein 2009). Además, a nivel de la comunidad se presenta mayor acceso a los recursos e intercambio de servicios (polinización y dispersión) aumentando la diversidad de especies (Stachowicz 2001; Callaway 2007; McIntire y Fajardo 2014).

En el Valle de Tehuacán-Cuicatlán, Valiente-Banuet y Verdú (2006, 2007, 2008), encontraron que en promedio el 96% de las especies vegetales de las comunidades se establecen por facilitación, lo cual da una idea de la importancia de este proceso en la organización de las comunidades. Con el uso de filogenias moleculares y la construcción de super árboles filogenéticos (Wikstrom et al. 2001; Webb et al. 2005), se ha encontrado que la facilitación ocurre entre especies distantes filogenéticamente (Valiente-Banuet y Verdú 2008). En cambio, especies emparentadas que suelen ser más similares fenotípicamente, ya que los rasgos del fenotipo que definen el nicho son conservados evolutivamente, tienden a competir (Valiente-Banuet y Verdú 2007; Verdú y Pausas 2007). Como resultado, la facilitación incrementa la diversidad filogenética de las comunidades (Valiente-Banuet y Verdú 2013).

Aún y cuando se ha mostrado para otros sistemas biológicos que la facilitación entre especies cercanas filogenéticamente puede cambiar a competencia a lo largo de la ontogenia de la interacción (Tielborger y Kadmon 2000; Miriti 2006; Soliveres et al. 2010), Valiente-Banuet y Verdú (2008), encontraron que el 57% de las asociaciones interespecíficas se mantienen hasta que los individuos facilitados llegan a la etapa adulta (Valiente-Banuet y Verdú 2008). Así, la acumulación de especies por debajo de una especie benefactora con respecto al tiempo contribuye a la formación de asociaciones interespecíficas bajo el dosel de una especie con lo cual se forman parches de vegetación con un número variable de especies. En este estudio nosotros hemos hipotetizado que en estos parches multiespecíficos constituidos por un número variable de especies se dan los mecanismos de la coexistencia, los cuales podrían estar dados por interacciones positivas entre los organismos como facilitación y mutualismo.

Información previa indica que la adecuación de plantas en los parches multiespecíficos es sensible al vecindario filogenético (Castillo et al. 2010). En otras palabras, a mayor distancia filogenética promedio (DFP) entre una especie focal y el vecindario del parche existe una mayor adecuación de especies focales (Castillo et al. 2010). El hecho de que la asociación entre especies se mantiene para más del 50% de las interacciones hasta la etapa adulta sugiere que el beneficio de la facilitación podría extenderse a todas las especies del parche. Hasta la realización de este trabajo, en todos los estudios realizados sobre facilitación el interés central fue determinar los beneficios que obtienen las especies facilitadas (Callaway 2007), siendo prácticamente nulos los estudios que demuestren incrementos en la adecuación de las especies benefactoras. Esta reciprocidad demostraría que la facilitación puede cambiar a mutualismo, lo cual contribuiría significativamente al entendimiento acerca de los mecanismos de la coexistencia en comunidades. A este respecto, diversos estudios señalan que, en el contexto de la facilitación, participan comunidades de microorganismos (van der Heijedn et al. 2008; van der Putten 2009). La mayoría de los estudios se han centrado en los efectos de la red común de micorrizas (RCM) en las plántulas facilitadas (Nara y Hogetsu 2004; Dickie et al. 2006), pero las nodrizas también están conectadas a la red y pueden recibir efectos positivos o negativos.

Los efectos positivos sobre las nodrizas explicarían el mantenimiento de las interacciones de facilitación hasta las etapas adultas en los parches de vegetación multiespecíficos y podrían ocurrir cuando las plantas facilitadas intercambian los beneficios a la nodriza a través de redes comunes de micorrizas. Los hongos micorrícicos arbusculares (HMA) pueden conectar varias plantas diferentes de la misma o de diferentes especies en una RCM (Newman 1988), y existe evidencia de transferencia de nutrientes y agua entre las plantas a través de redes micorrícicas (Giovanneti et al. 2004; Selosse et al. 2006; Egerton- Warburton et al. 2007; Mikkelsen et al. 2008; Simmard et al. 2012; Montesinos-Navarro et al. 2017; Montesinos-Navarro et al. en prensa), pero la magnitud y por lo tanto la importancia ecológica para el desempeño de la planta todavía se debate (Van der Heijden y Horton 2009; Bever et al. 2010). Los beneficios proporcionados por los hongos micorrícicos a las plantas son bien conocidos, contribuyendo hasta un 90% de la absorción de P de las plantas, mejorando la productividad (Jakobsen et al. 1992; Van der Heijden et al. 1998, 2006), proporcionando tolerancia a la sequía (Augé 2001), aumentando la tolerancia a patógenos (Sikes et al. 2009; Veresoglou y Rillig 2012), acelerando la descomposición y adquisición de N de la materia orgánica (Hodge et al. 2001), así como incrementando la fijación de N_2 por leguminosas (Turk et al. 2008). A cambio, las plantas proporcionan hasta el 40 % de compuestos de carbono a los hongos micorrícicos y a todos los microorganismos (Smith y Read 2008).

Mimosa luisana Brandegeese es una especie nodriza clave en el Valle de Zapotitlán, que es capaz de establecerse en sitios abiertos y mejorar el microhabitat debajo de su copa, permitiendo el establecimiento de un gran número de especies de la comunidad (90%), dando como resultado parches discretos (Cerón 2015), incluso filogenéticamente más diversos que lo esperado por el azar, es decir, parches constituidos por especies pertenecientes a una gran variedad de linajes filogenéticos (Valiente-Banuet y Verdú 2007; Castillo et al. 2010; Cerón 2015). *M. luisana* es leguminosa que está en simbiosis con bacterias fijadoras de N de la familia Rhizobiaceae, alfaproteobacterias del género *Ensifer* (Sprent y Gehlot 2010; Bontemps et al. 2016). Estas bacterias forman nódulos en las raíces y mediante enzimas (complejo enzimático nitrogenasa) y utilizando como energía el malato y fumarato producido por la planta, reducen el N_2 a amonio (NH_4) para ser liberado a la planta y forme compuestos orgánicos nitrogenados. También el amonio puede ser asimilado por las bacterias y liberado en forma de alanina (Dilworth et al. 2008). A través de esta relación la planta puede obtener hasta el 90% de N que requiere (Franche et al. 2009; Richardson et al. 2009).

En la red de interacciones planta-micorriza, *M. luisana* es un nodo hiperconectado a un gran número de micorrizas que a su vez conectan a otras especies de plantas de los parches (Montesinos-Navarro et al. 2012). Se ha mostrado que las micorrizas arbusculares pueden ayudar a descomponer y movilizar el N en forma de amonio y nitrato a la planta (Hodge et al. 2001). El micelio externo de las micorrizas absorbe el N inorgánico (NO_3^- y NH_4) del suelo y es asimilado vía nitrato reductasa, convirtiéndose posteriormente en Arginina, translocándose al micelio interno para degradarse y liberar urea y/o ornitina para formar aminoácidos o NH_4 y ser utilizados por la planta (Govindarajulu et al. 2005; Jin et al. 2005).

Por lo anterior las interacciones multiespecíficas y multitróficas son cruciales en el ensamblaje de las comunidades y esta tesis centra su análisis en entender los mecanismos de coexistencia que ocurren en los parches de vegetación en una comunidad de "Tetechera" de *Neobuxbaumia tetetzo,* donde *M. luisana* es la principal nodriza.

Consecuentemente esta tesis analiza primeramente la existencia de mutualismos facultativos como base de la coexistencia de especies y la importancia de las interacciones multiespecíficas en vecindarios filogenéticamente diversos mediante un experimento de campo evaluando el desempeño (éxito reproductivo) de individuos de *M. luisana* creciendo solos y asociados en parches con diferente vecindario filogenético. Los resultados de esta investigación se presentan en el primer capítulo titulado "Nurses experience reciprocal fitness benefits from their distantly related facilitated plants", publicado en la revista Perspectives in Plant Ecology, Evolution and Systematics 16: 228–235 (2014).

En el segundo capítulo se pone a prueba la hipótesis de que el mutualismo se da por la participación de redes de hongos micorrícicos arbusculares que conectan a *M. luisana* con sus plantas facilitadas. Probamos experimentalmente en condiciones de campo si la red común de micorrizas establecida entre *M. luisana* y sus especies de plantas facilitadas median el aumento del desempeño de la primera. Se aplicó fungicida eliminando las micorrizas, es decir, desconectando a *M. luisana* de la red y se comparó su efecto sobre la cantidad y calidad de la producción de semillas como medidas de los diferentes componentes de adecuación de individuos de *M. luisana* creciendo solos y asociados (parches). Los resultados de este estudio se presentan en el artículo titulado "Nurse plants benefit from facilitative interactions through mycorrhizae", que está siendo revisado en la revista *Fungal Ecology*.

En el tercer capítulo, se pone a prueba la hipótesis de la transferencia de nutrientes desde la especie facilitadora a las especies facilitadas, para entender los casos en los cuales especies benefactoras y beneficiarias se mantienen asociadas hasta que llegan a la etapa adulta. Para ello se hizo un experimento de campo en el cual se usaron isótopos estables de nitrógeno que fueron absorbidos por las hojas de *M. luisana*, una especie fijadora de nitrógeno para posteriormente ser detectado en plantas adultas facilitadas, y evaluar hasta qué punto la red común de micorrizas median la transferencia de nutrientes. Se marcaron a los individuos de *M. luisana* con urea enriquecida con ^{15}N y se cuantificó la cantidad de ^{15}N transferida a sus plantas adultas facilitadas en parches con (eliminando los hongos micorrícicos) y sin fungicida (con hongos micorrícicos). Los resultados se presentan en el artículo titulado "Soil fungi promote nitrogen transfer among plants involved in long-lasting facilitative interactions", publicado en la revista Perspectives in Plant Ecology, Evolution and Systematics 18: 45–61 (2016).

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CAPITULO I

Nurses experience reciprocal fitness benefits from their distantly related facilitated plants

Perspectives in Plant Ecology, Evolution and Systematics 16 (2014) 228–235

Contents lists available at ScienceDirect

Perspectives in Plant Ecology, Evolution and Systematics

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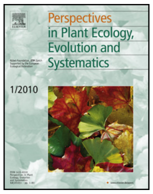

Research article

Nurses experience reciprocal fitness benefits from their distantly related facilitated plants

Lugui Sortibrán[a], Miguel Verdú[b], Alfonso Valiente-Banuet[a,c,*]

[a] Departamento de Ecología de la Biodiversidad, Instituto de Ecología, Universidad Nacional Autónoma de México, A. P. 70-275, C. P. 04510 México, D. F., México
[b] Centro de Investigaciones sobre Desertificación (CIDE, CSIC-UV-GV), Carretera Moncada – Náquera, Km. 4.5, 46113 Valencia, Spain
[c] Centro de Ciencias de la Complejidad, Ciudad Universitaria, Universidad Nacional Autónoma de México, 04510 México, D. F., México

ARTICLE INFO

Article history:
Received 7 March 2014
Received in revised form 8 July 2014
Accepted 10 July 2014
Available online 17 July 2014

Keywords:
Facilitation
Mutualism
Phylogenetic neighborhood
Plant–plant interactions
Positive interactions
Species coexistence

ABSTRACT

It is well known that many plants benefit from growing beside a nurse plant of another species, but the possibility that the nurse also benefits has been rarely studied. We hypothesize that positive interactions are maintained not only because of the recruitment benefits for the facilitated plants but also because of fitness benefits for the nurse plant. We tested this hypothesis by comparing seed production, seed predation and seed viability of a dominant nurse plant species (*Mimosa luisana*) when growing alone and in patches surrounded by its facilitated species. We also tested whether fitness of the nurse species is dependent on the phylogenetic neighborhood formed by their facilitated species using an analysis that accounted for the abundance and pairwise phylogenetic similitude of all species in each patch. Nurses growing associated to their facilitated species produced more seeds (1.86 times) and these seeds were more viable (1.47 times) than those of nurses growing alone. Seed predation did not alter these fitness differences. Seed number and viability increased in phylogenetically diverse neighborhoods. We conclude that distantly related partners are more likely to cause reciprocal increases in fitness, and that such effects contribute to species coexistence.

Introduction

The recent interest in the role of facilitation in plant communities has helped redress an undue emphasis upon antagonistic interactions, though some crucial topics are still poorly covered (Brooker et al., 2008). Thus, while the relationship between facilitation and competition has been extensively analyzed, the relationship between facilitation and mutualism has practically been unexplored (Bronstein, 2009).

Under the dominant influence of the competition paradigm, many studies using pairs of species have shown that facilitation may turn gradually into competition as plants grow larger (Miriti, 2006). For example, Miriti et al. (2001) showed a negative impact of adult neighbors upon population growth rates of *Ambrosia dumosa.* Asymmetric responses in the facilitation–competition balance have also been found, with consequences that were harmful for the facilitated species but harmless for the nurse (Verdú et al., 2004). Similarly, competitive interactions have been found to prevail in many observational and experimental studies that considered several facilitated species growing with a nurse plant (Schöb et al., 2014). However, a search of the literature indicates that in semiarid communities positive interactions are more likely to persist. Thus, a shift from facilitation toward competition was reported in only 43% of cases, while facilitative effects persisted in 57% of cases (Valiente-Banuet and Verdú, 2008).

It is generally supposed that species coexistence is primarily a consequence of niche differentiation, and that this tends to be greater between more distantly related species (Valiente-Banuet and Verdú, 2007; Soliveres et al., 2010, 2012 but see Cahill et al., 2008; Mayfield and Levine, 2010). In many ecosystems, facilitation leads to the formation of discrete, multi-specific vegetation patches surrounded by open space, although the significance of these has been largely overlooked (Prentice and Werger, 1985; Eccles et al., 1999; Castillo et al., 2010). Furthermore, the few experimental studies performed in such vegetation have shown that facilitated species benefit more in phylogenetically diverse neighborhoods

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[*] Corresponding author at: Departamento de Ecología de la Biodiversidad, Instituto de Ecología, Universidad Nacional Autónoma de México. A. P. 70-275, C. P. 04510 México, D. F., México. Tel.: +52 5556229010.
E-mail address: avalib@gmail.com (A. Valiente-Banuet).

http://dx.doi.org/10.1016/j.ppees.2014.07.001
1433-8319/

(Castillo et al., 2010). Similarly, the richness of facilitated species tends to have positive effects on nurse fitness, indicating that the costs and benefits of harboring associated species depend on the composition of the plant assemblage (Schöb et al., 2014). In such cases, we might expect any benefits to the nurse species to depend upon its evolutionary relationships to its neighbors. Improved plant performance in phylogenetically diverse neighborhoods has been interpreted as indicating reduced competition between distantly related species (Castillo et al., 2010). However, it is tempting to hypothesize that indicates mutualism between distantly related plants (Valiente-Banuet and Verdú, 2013). For example, a distantly related plant can be more helpful to a neighbor if it provides functionally complementary fungi to the mychorrizal network (Beltrán et al., 2012; Montesinos-Navarro et al., 2012a).

A demonstration of non-random association of species together with physiological and growth benefits for the nurse species (Pugnaire et al., 1996) would provide valuable evidence about the mutualistic nature of facilitation. However, to demonstrate such a mutualism, it is ultimately necessary to measure a fitness component like reproductive success and compare it with the fitness of individuals that are not involved in facilitation (Bronstein, 2009). Fitness benefits for associated individuals may arise via different mechanisms, including microclimatic amelioration, associational defense and shared mutualists (Callaway, 2007; Bronstein, 2009; Beltrán et al., 2012). For example, nurses might benefit from their facilitated plants if these reduce direct insolation, or reduce soil erosion, or increase fertility by providing organic matter (Pugnaire et al., 1996). Associational defenses and shared mutualisms are biotic mechanisms to improve the microenvironment where plants coexist. Mycorrhizal fungal networks have shown to provide these functions in the facilitation interactions because nurse and facilitated plants can help each other by sharing mycorrhizal fungi that enhances nutrient supply and protect against pathogens (Selosse et al., 2006; Van der Heijden and Horton, 2009).

Here we hypothesize that positive interactions within a vegetation patch are maintained not only by providing recruitment benefits for facilitated plants but also by improving the performance of nurse species. We specifically determined whether the shrub *Mimosa luisana*, a key nurse species forming multi-specific patches through the facilitation of 86% of species in Mexican deserts (Supplementary Material Fig. S1), has higher reproductive success when growing in these patches than when it grows alone. We subsequently tested whether the fitness benefits for *M. luisana* increase with increasing phylogenetic distance of the facilitated species.

Materials 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 of the Tehuacán-Cuicatlán Valley in the state of Puebla, 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). Specifically, the study site is located inside the Botanical Garden “Helia Bravo Hollis”, a natural unmodified protected area located approximately 30 km south of Tehuacán city in which the vegetation is a xeric shrubland dominated by the columnar cactus *Neobuxbaumia tetetzo*, and the species such as *M. luisana, Mascagnia seleriana, Ipomoea arborescens, Aeschynomene compacta, Caesalpinia melanadenia, Calliandra eryophylla, Zapoteca formosa, Senna wislizenii, Agave marmorata*, *Agave macroacantha*, *Jatropha neopauciflora*, among other taxa (Valiente-Banuet et al., 2000). Most of the species that have been recorded in this community (48 out of 56) appear to depend upon *M. luisana* plants for recruitment. These include species of several functional groups – shrubs, succulents plants such as *Agave* and cacti, perennial climbing vines, and perennial herbs (Verdú and Valiente-Banuet, 2008) – which together form discrete patches of vegetation. The area occupied by a vegetation patch ranges from 1 to 5 m^2 and corresponds to the vertical projection of the canopy of an adult individual of *M. luisana*. We regard facilitated plants as being those restricted to the subcanopy of the focal *M. luisana* individuals. In addition, isolated individuals of *M. luisana* can also be found.

M. luisana is a deciduous spiny shrub reaching heights up to 2.2 m. Plants reach reproductive maturity when they are ca. 0.80 m tall. The pink, hermaphrodite flowers occur in spike-like inflorescences and are produced during July at the beginning of the rainy season. Seeds are produced within a spiny brown legume. Seeds have a hard, wax-covered coat and scarification is needed to break dormancy and germination occurs after few days (Camargo-Ricalde et al., 2004). Seed predation rates by the bruchids *Acanthoscelides mexicanus*, *A. chiricahuae*, and *Stator pruininus* range between 30 and 75% (Camargo-Ricalde et al., 2004; Romero-Nápoles et al., 2005). Pods and seeds are eaten by mammals such as horses (*Equus caballus*), donkeys (*Equus africanus*) and goats (*Capra hircus*). At present goats have been eliminated from the study area, as well as the white-tailed deer (*Odoicoleus virginianus*) considered as the native main possible seed disperser. Although a high proportion of seeds consumed by goats, the remaining seeds germinate in a higher proportion after gut passage than control treatment (47.5 vs. 5.83% respectively; Giordani, 2008). Goats, horses, and donkeys are efficient dispersal agents for the seeds of *M. luisana*, carrying seeds considerable distances and depositing their feces in open areas that are suitable for germination.

Fitness estimation

Three components of *M. luisana* fitness were estimated: (i) seed production, (ii) seed predation and (iii) seed viability. Seed production was estimated by counting the total number of seeds produced by the shrub *M. luisana* in patches with and without neighbors. A total of 90 reproductive *M. luisana* individuals (>80 cm height) growing alone ($N = 25$) and growing associated to other species ($N = 65$) were randomly selected. This unbalanced design corresponded to the natural distribution of isolated and associated individuals. The size of shrubs was estimated as the volume of an inverted cone with an elliptical base by measuring the height and two perpendicular diameters of the canopy cover (Supplementary Material Table S1). Seed predation was estimated by counting the number of seeds with the exit hole done by bruchids in random samples of 300 seeds obtained from each *M. luisana* individual growing without or with neighbors ($N = 17$ and $N = 29$ respectively). Seed viability was estimated as the proportion of non-preyed seeds that were able to germinate. Seeds were scarified with sandpaper to reduce the thickness of the testa, and germinated in Petri dishes with filter paper at 25 °C in 12 h. light/dark in a growth chamber.

A potentially confounding factor in the relationship between nurse fitness and coexistence with facilitated plants may be microclimatic variation. For example, a very rich microhabitat may independently support the establishment of multiple species and enhance *M. luisana* fitness. For this reason, it is important to ensure that there is a nursing effect of *M. luisana* on the rest of species and the association is not merely the response of plants to a resource-rich patch. To check that this was not the case, we randomly sampled soils to a depth between 0–10 cm below the canopy of

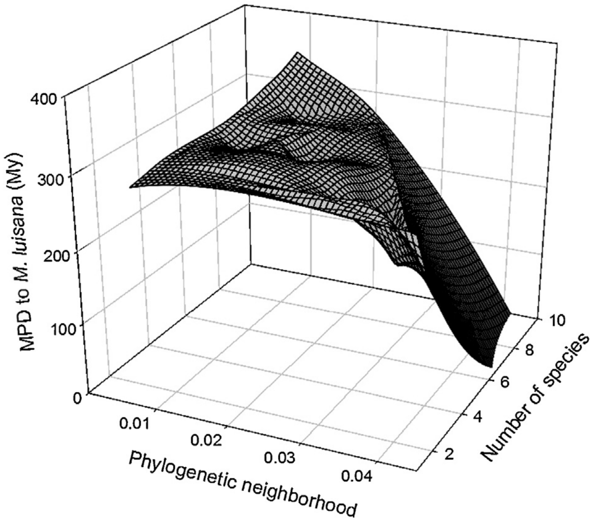

Fig. 1. Relationship between the phylogenetic neighborhood metrics (Pillar and Duarte, 2010) and both the number of species and the mean phylogenetic distance of associated species to *Mimosa luisana* in each vegetation patch. High phylogenetic neighborhood values occur when the abundance of species closely related to *M. luisana* in the patch is high whereas low values occur in patches with distantly-related species to *M. luisana*.

20 young *M. luisana* plants (ca. 10 cm height) and compared it to soil collected in their neighboring gaps.

To test whether nurse benefits could result from micro-environmental amelioration produced by an increasing number of their facilitated plants, we measured soil fertility (pH, electrical conductivity, organic matter, total N, total P and total K, olsen P, N-NO_3^-, N-NH_4^+, $CaCO_3$) physicochemical structure (cation exchange capacity, % sand, silt and clay) and water availability (gravimetric humidity and water holding capacity) in soils of 14 patches and correlated with the number of facilitated species in the patch (0–7).

Phylogenetic neighborhood calculations

The phylogenetic neighborhood of *M. luisana* in each patch was characterized following the framework for meta-community analysis of phylogenetic structure developed by Pillar and Duarte (2010). In contrast to other widely used metrics such as Mean Phylogenetic Index (MPD) or Mean Nearest Neighbor Distance (MNND), Pillar and Duarte metrics incorporates the identity of coexisting plant species. For example, a patch where *M. luisana* coexists with both a distant and a closely related species (300 and 10 My respectively) will have the same MPD than a patch where *M. luisana* coexist with two species with intermediate (150 and 160 My respectively) phylogenetic distances. However, Pillar and Duarte metrics will score each patch with different values by characterizing the phylogenetic structure of a given patch taking into account the abundance and the pairwise phylogenetic similitude of each species with the rest of the species in the community. A matrix (P matrix) containing the composition of the patches is calculated by fuzzy weighting of species abundance by the species phylogenetic similarities (see Pillar and Duarte, 2010 for the mathematical explanation). Each element of the P matrix represents the phylogenetic neighborhood of a focal species in a given patch. We used the elements of the P matrix corresponding to *M. luisana* to characterize its phylogenetic neighborhood in each patch. This metric of phylogenetic neighborhood is correlated with both the species richness and the mean phylogenetic distance of *M. luisana* to all its neighbors in the patch (Fig. 1). More specifically in our system, vegetation patches with many closely-related species to *M. luisana* have high scores of phylogenetic neighborhood while patches with distantly-related species to *M. luisana* have low values (Fig. 1).

The phylogenetic similarity matrix was obtained from the community phylogeny generated with the help of the program Phylomatic as implemented in Phylocom 4.2 (Webb et al., 2008) and BEAST 1.5.4 (Drummond and Rambaut, 2007). The topology of the community phylogeny was obtained with Phylomatic by matching the family names of our study species with those contained in a backbone phylogeny, which is the megatree based on the work of the Angiosperm Phylogeny Group 3 (Stevens, 2005). We then resolved the phylogenetic relationships at the species level of the Fabaceae, Cactaceae and Agavaceae families based on published phylogenies of Good-Avila et al. (2006), Simon et al. (2009), and Hernández-Hernández et al. (2011). Our final tree was fully resolved with the exception of two polytomies (Supplementary Material Fig. S2). We simultaneously resolved these polytomies and adjusted branch lengths with the help of BEAST (Drummond and Rambaut, 2007) and the PolytomyResolver script (Kuhn et al., 2011). This branch length adjustment procedure is based on an evolutionary, birth-death model and has been shown to be more realistic than traditional non-model-based approaches, like that used by the bladj algorithm in phylocom (Webb et al., 2008) which assign branch lengths by evenly distributing the undated nodes between the known parent age and the known daughter age. The PolytomyResolver script indicates to the BEAST program the chronological and topological constraints as well as the specifications of a birth–death tree prior. We defined chronological constraints for 42% of the node ages on the basis of published chronograms (Wikstrom et al., 2001; Good-Avila et al., 2006; Simon et al., 2009; Arakaki et al., 2011; see Supplementary Material Fig. S2 for node ages), and the remaining nodes were left to be dated by BEAST using the default settings specified in the PolytomyResolver script. We ran Markov Chain Monte Carlo (MCMC) analyses for 10^6 iterations, sampling trees every 10^3 iterations, discarded a 25% burnin, and randomly selected 100 fully-resolved dated trees. We used these 100 trees in the subsequent analysis to account for the topological and chronological uncertainty in our phylogenetic tree.

Data analyses

We first compared the three fitness components for *M. luisana* individuals growing alone and in association with other species using a Bayesian Generalized Linear Model with different distributions of errors (Poisson for seed production and binomial for seed viability and seed predation). The size of each *M. luisana* individual was also included in the model because large individuals may have more seeds than small ones.

Second, we tested whether the three *M. luisana* fitness components depended on its phylogenetic neighborhood as well as on its size by means of the same Bayesian Generalized Linear Models. Bayesian models allow different sources of uncertainty to be accommodated in the model. More specifically, we integrated topological and chronological uncertainty associated with phylogenetic reconstructions. To do this, we ran 100 Bayesian GLM models with the phylogenetic neighborhood scores calculated from the 100 phylogenetic trees. Then, we integrated over the posterior samples by drawing 1000 random samples across models (Longdon et al., 2011). The models were run with the help of MCMC techniques as implemented in the MCMCglmm package for R (Hadfield, 2010; R Development Core Team, 2011). We used the default priors and ran 13,000 MCMC iterations with a burn-in period of 3000 iterations. Convergence of the chain was tested by means of an autocorrelation statistic. The statistical significance of the factors in the model was

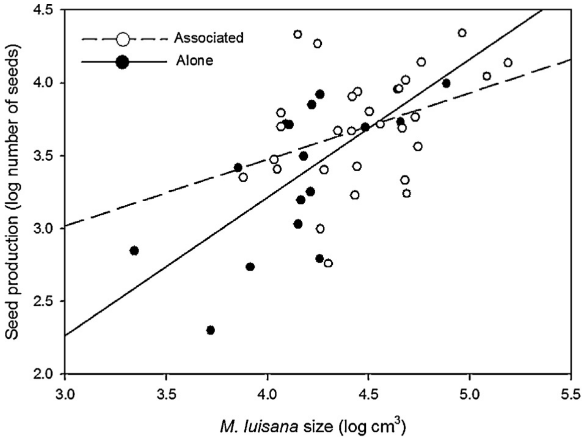

Fig. 2. *Mimosa luisana* shrubs produced more seeds when growing associated with other species (dashed line) than when growing alone (solid line) in small and medium sized shrubs but not in large shrubs.

estimated by calculating the 95% credible interval of their posterior distribution. Means are shown with standard errors throughout the manuscript.

Results

Microhabitat characteristics

Soils samples taken from under young *M. luisana* individuals (ca. 10 cm height) were not significantly different from those taken in open areas for any soil fertility measures (Supplementary Material Table S2). This suggests that improved soil conditions under *M. luisana* adults was not a cause of initial recruitment in the most fertile microhabitats.

The microhabitat amelioration produced by *M. luisana* adults did not change with the number of facilitated species in the vegetation patch (Supplementary Material Table S3)

Fitness estimates of M. luisana with and without neighbors

The mean number of seeds produced by *M. luisana* shrubs associated with other species was significantly higher (1.86 times greater) than by shrubs growing alone (Table 1; Fig. 2). The number of seeds increased significantly with shrub size and there was a significant although weak negative interaction between shrub size and the association condition of the shrub (associated vs. alone) (Table 1). This interaction occurred because the seed production benefits of growing associated with other species was evident in small and medium shrubs but not in large ones (Fig. 2).

Seed predation rate did not differ between individuals of *M. luisana* living with and without neighbors (0.22 ± 0.01 and 0.17 ± 0.03 respectively; Table 1). Seed predation was also independent of shrub size and the interaction between association condition and size (Table 1).

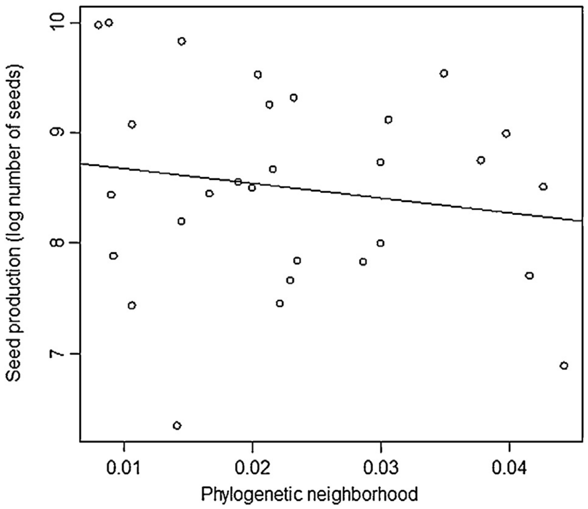

Fig. 3. Number of seeds produced by *Mimosa luisana* as a function of its phylogenetic neighborhood. The fitted line is drawn to illustrate the trend, but the appropriate statistical model can be seen in the Results section.

Mean seed viability differed significantly for *M. luisana* individuals growing with and without other species, being 1.47 times higher for shrubs associated with other plants (0.62 ± 0.03 and 0.42 ± 0.04 respectively; Table 1). This difference was independent of shrub size and the interaction between association condition and size Table 1).

Taking together these results, it can be estimated that *M. luisana* living with neighbors have 2.7 times (1.86 × 1 × 1.47) the fitness of individuals living alone.

Fitness estimates of M. luisana as a function of its phylogenetic neighborhood

The mean number of plant species associated with *M. luisana* was 3.7 ± 0.3 (range 1–10) species, and the mean number of individual plants was 6.8 ± 0.9 (range 1–42). *M. luisana* did not coexist with close relatives such as *A. compacta*, *Z. formosa*, *C. melanadenia* and *C. eryophylla* unless species of other families (Cactaceae, Asteraceae, Agavaceae, Acanthaceae, Bromeliaceae) were also present in the patch.

The number of seeds produced by *M. luisana* growing in vegetation patches varied according to the phylogenetic composition of the patch (Fig. 3), being high in patches with distantly related species and lower in patches harboring many closely-related neighbors (Fig. 3; Table 2). Interestingly, this correlation was independent of shrub size as there was no significant interaction between phylogenetic neighborhood and size (Table 2), indicating that seed production dependence on phylogenetic neighborhood was similar across all the shrub sizes.

Seed predation of *M. luisana* growing in vegetation patches was independent on its phylogenetic neighborhood, its size and

Table 1
Bayesian Generalized Linear Models testing for the effects of growth condition (without or with neighbors) and shrub size on three different fitness components of *M. luisana* individuals (seed production, seed predation and seed viability). Post-mean estimates are shown with their 95% credible intervals.

	Seed production	Seed predation	Seed viability
Intercept	3.64 [2.00, 5.17]	−1.23 [−1.77, −0.76]	−0.04 [−1.09, 0.21]
Condition	2.65 [0.91, 4.72]	0.45 [−0.18, 1.05]	0.96 [0.06, 1.77]
Size	1.1e−04 [4.3e−05, 1.8e−04]	1.67e−05 [−6.46e−07, 3.42e−05]	2.53e−06 [−2.05e−05, 2.77e−05]
Condition X size	−8.3e−05 [−1.5e−04, −1.1 e−05]	−1.73−05 [−3.63e−05, 1.83e−06]	−2.86e−06 [−3.18e−05, 1.96e−05]

Table 2
Bayesian Generalized Linear Model testing for the effects of phylogenetic neighborhood and shrub size on three different fitness components of *M. luisana* individuals (seed production, seed predation and seed viability). The model accommodates the phylogenetic uncertainty by integrating over the posterior samples of 100 models in which the phylogenetic neighborhood were calculated from 100 different phylogenetic trees. High phylogenetic neighborhood values occur when the abundance of species closely related to *M. luisana* in the patch is high. Post-mean estimates are shown with their 95% credible intervals.

	Seed production	Seed predation	Seed viability
Intercept	9.39 [6.61, 12.3]	−0.38 [−2.4, 2.0]	2.14 [0.80, 3.54]
Phylo neighb	−1.14e02 [−2.24e02, −1.54e01]	25.9 [−56.5, 121.1]	−47.89 [−111.85, −2.43]
Size	−3.14e−05 [−1.2e−04, 4.4e−05]	1.2e−05 [−4.7e−05, 7.1e−05]	−2.42e−05 [−6.77e−05, 1.11e−05]
Phylo neighb *size	2.7e−03 [−7.8e−04, 6.5e−03]	−7.2e−04 [−3.5e−03, 2.1e−03]	1.0e−03 [−6.98e−04, 2.83e−03]

from the interaction between phylogenetic neighborhood and size (Table 2).

Seed viability was high in patches with distantly related neighbors and lower in patches harboring many closely-related neighbors (Fig. 4). This relationship was significant and independent of shrub size. Also, there was no significant interaction between phylogenetic neighborhood and size (Table 2), indicating that seed viability dependence on phylogenetic neighborhood was similar across all the shrub sizes.

Discussion

Our study shows that the fitness of the key nurse in this semi-arid community, *M. luisana*, is higher when growing associated with their facilitated species than when growing alone. Contrary to the common finding that facilitation turns into competition with time (Miriti, 2006), we show that *M. luisana* continues to benefit in terms of its reproductive output, although the effect is less strong for large plants. The appearance of competitive effects only under the largest *M. luisana* shrubs is consistent with the results described for the columnar cactus *Neobuxbaumia tetezo* recruiting beneath *M. luisana* in our study system (Flores-Martínez et al., 1994). However, by moving beyond pairwise interactions, we find that the fitness of the nurse *M. luisana* is positively affected by the presence of several, distantly-related neighbors, and that this benefit applies for shrubs of all sizes. These results have implications for understanding species coexistence in communities by extending the concept of facilitation, which is usually considered to be an asymmetrical effect, to include mutualism, where both facilitated and nurse species benefit.

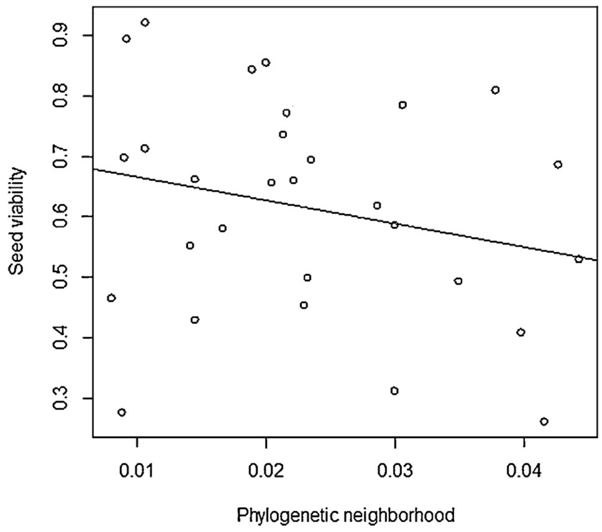

Fig. 4. Seed viability of *Mimosa luisana* as a function of its phylogenetic neighborhood. The fitted line is drawn to illustrate the trend, but the appropriate statistical model can be seen in the Results section.

In our system, the nurse *M. luisana* establishes in the open ground and ameliorates the microhabitat, enabling other species to establish and ultimately resulting in the formation of multi-specific patches (Valiente-Banuet and Verdú, 2008). Facilitated species increase their fitness through high seedling survival under *M. luisana*, where temperatures and evaporative demands are lower than in open ground and where no seedlings can survive (Valiente-Banuet and Ezcurra, 1991). Here, we demonstrate that fitness of nurse plants is also enhanced by the presence of neighbors, suggesting that plants may be helping each other. Nurses might be benefitting from the microclimatic amelioration produced by their facilitated plants, as occurred for example with *Retama sphaerocarpa* nurses benefitting from the soil improvement produced by their facilitated plants, ultimately resulting in higher biomass, total nitrogen content and higher water potentials (Pugnaire et al., 1996). However, in our study the number of facilitated species had no influence upon soil fertility or water availability, indicating that it was not these abiotic factors that caused the observed fitness effects upon *M. luisana*. However, micro-environmental amelioration via biotic factors can also be produced. Growing evidence exists that mutual benefit among neighboring plants may occur through the establishment of a common mycorrhizal network that enables resources to be shared (Selosse et al., 2006; Simmard et al., 2012). In our study area, this network is not random but is phylogenetically structured in such a way that closely related fungi tend to interact with the same plant species (Montesinos-Navarro et al., 2012b). Among all the plants in the network, *M. luisana* is a key species acting as a mycorrhizal and nutrient "resource island" from which a range of plants associated with it may benefit (Camargo-Ricalde and Dhillion, 2003; Montesinos-Navarro et al., 2012b). Once the mycorrhizal network is established, *M. luisana* may benefit from its facilitated plants by sharing their resources (Selosse et al., 2006). Unpublished experiments with fungicides show that the disruption of this mycorrhizal network significantly decreases the number of seeds (from 1014 ± 241 to 511 ± 132) and the leaf dry-matter content (from 18.1 ± 0.2% to 17.1 ± 0.3%) of *M. luisana* (L. Sortibrán, M. Verdú, M. & A. Valiente-Banuet, unpublished). These results suggest that resource sharing among plants is the mechanism underlying the higher fitness of *M. luisana* in vegetation patches. We have also shown that growing with neighbors allows *M. luisana* not only to produce more seeds but also more viable seeds, which is consistent with higher resource availability provided by multiple neighbors. The demographic fate of these seeds is not jeopardized by seed predators because the predation rate is unaffected by the presence of neighbors. The main predators of *M. luisana* seeds are bruchids, which feed exclusively upon legumes (Johnson and Siemens, 1995; Camargo-Ricalde et al., 2004; Romero-Nápoles et al., 2005); since closely related legumes were almost absent from the vegetation associated with *M. luisana*, they had no effect upon predation rates.

In short, contrary to the commonly reported outcome that facilitation turns into competition with time (Miriti, 2006; Schöb et al., 2014), we find mutually positive interactions that allow the nurse shrub to increase its fitness. But not all the neighbors are equivalent for a given plant species. For example, the performance of facilitated seedlings is dependent on the identity of the nurses (Castillo and Valiente-Banuet, 2010; Castillo et al., 2010). Such specificity has a strong phylogenetic component because closely related facilitated species tend to recruit under the same nurses, indicating that seedlings completely depend on the regeneration niche provided by specific nurse species (Verdú et al., 2010). Of these, *M. luisana* is a hub in the facilitation network that may facilitate up to 48 species in the community we studied (Verdú and Valiente-Banuet, 2008). We found that the fitness of the nurse *M. luisana* was higher when the neighboring plants were distantly related to it. This demonstrates that lifetime fitness is affected by the species that become established. As most mutualisms, facilitation would be a diffuse mutualism involving multiple species interacting (Palmer et al., 2003). Contrary to the traditional approach to mutualisms, which considers interactions between pairs of species in isolation, our results suggest that fitness is affected by multiple partnerships, as has also been shown in other systems (Palmer et al., 2010). To unambiguously assess that facilitation is a mutualism, the genetic basis of relevant traits associated to facilitation should be identified. Although we do not have such evidence, promising new evidence indicates that phenotypic differences in facilitative effects of a nurse species have a genetic basis (Michalet et al., 2011).

Three non-exclusive mechanisms potentially explaining why distantly related species enhance fitness of *M. luisana* are (i) competition release, (ii) resource sharing, and (iii) enemy escape. Competition is reduced when niches of coexisting species do not overlap. As functional and ecological similarities are usually shaped by patterns of common ancestry, phylogenetic unrelatedness is a good proxy of ecological differentiation (Wiens and Graham, 2005; but see Cahill et al., 2008; Mayfield and Levine, 2010). Consequently, more distantly related species in the vegetation patch would reduce niche overlap, reducing at the same time competition (Valiente-Banuet and Verdú, 2008). In our case, the reduction of competition in phylogenetically diverse neighborhoods may allow *M. luisana* to increase its reproductive output. Competition release in the presence of distantly related partners may also explain why close relatives of *M. luisana* only coexisted with it in the presence of other, unrelated species. In an adjacent community, Castillo et al. (2010) also reported indirect effects through distantly related species to explain the coexistence of close relatives within the same vegetation patch. However, it should be noted that competition release cannot explain why the lowest fitness was detected in nurses growing alone. As our results clearly indicate that living with other species enhances nurse fitness, we should move from explanations based only upon competition to consider the possibility that plants are helping each other.

The idea that resource sharing can be maximized in phylogenetically diverse neighborhoods would explain why *M. luisana* reproduced best when associated with less related species. There is growing evidence that belowground process involving plant–mycorrhizal interactions might be operating in the facilitation processes (Van der Heijden and Horton, 2009; Van der Putten, 2009; Selosse et al., 2006; Montesinos-Navarro et al., 2012a,b; Simmard et al., 2012). Specifically, the existence of a phylogenetically and therefore functionally diverse rhizosphere has been shown to contribute to a high plant productivity (Bever, 2002; Maherali and Klironomos, 2007). Interestingly, *M. luisana* belongs to a family that benefits more than most others from mycorrhizal fungi, because of the high phosphorus requirement of leguminous plants to support symbiotic nitrogen fixation (Scheublin et al., 2007). In the extremely low-resource environment we studied (e.g. low rainfall and low P content in soils), the benefits from sharing a mycorrhizal network with facilitated, distantly-related species may be particularly high.

Enemies may also be avoided in a diverse phylogenetic neighborhood. Although we have shown that this is not the case for seed predators, other enemies like pathogens may infect a set of more closely related hosts because of their shared phylogenetic predilections (Webb et al., 2006; Yguel et al., 2011). Therefore, phylogenetically distant plant species can promote an increase of fitness by decreasing the presence of plant pathogens (Van der Putten, 2009). Future research on the effects of other enemies on the fitness of the *M. luisana* living in different phylogenetic neighborhoods will help to understand the selective pressures favoring the reciprocation of benefits between plant species we have detected.

Although these ideas seem plausible, the observational nature of our study means that we cannot exclude the possibility that other factors were responsible for the observed benefits to *M. luisana* of growing in species-rich patches. In principle, one could study the fitness effects of experimentally removing neighbors. However, below-ground interactions, which may be responsible for the observed patterns, might respond very slowly to such perturbations, with the established soil community persisting for years or even decades after individual plants had been removed (Van der Putten, 2009). One potentially confounding factor in the relationship between *M. luisana* seed production and coexistence with other plants is microclimatic variation. For example, *M. luisana* colonizing stressful microclimates would have limited both its seed production and the coexistence with other species due to resource competition. In contrast, in less stressful microclimates seed production may be higher and the coexistence with other species allowed due to the lack of resource competition. However, we have discarded this confounding effect by showing that microhabitat amelioration is a direct effect, and not the cause, of nurse establishment. Furthermore, that confounding effect could not explain why nurse fitness increases with phylogenetic distance to its neighbors.

Community ecology implications

In contrast to theoretical models showing that communities driven by positive interactions tend to extinction (Kefi et al., 2008 and references therein), our results support the view that mutualistic interactions minimize competition and increase biodiversity (Wilson and Nisbet, 1997; Okuyama and Holland, 2007; Bastolla et al., 2008). We have shown that living in diverse neighborhoods produces fitness benefits for the nurse plants, though we know little about the demographic consequences of this effect. One possible consequence might be mediated through increased herbivory, since seeds of *M. luisana* germinate more readily after passage through the gut of a herbivore such as a goat (Giordani, 2008). Since these mammals defecate in open spaces and away from the mother plant, the only suitable sites for seedling establishment, they act as efficient seed dispersers, which may have a positive demographic effect on *M. luisana* populations. Patch-specific matrix models to test the influence of the phylogenetic neighborhood on the demography of nurse plants (Miriti et al., 2001) could help to understand the evolutionary potential of facilitation as a mechanism allowing the populations to escape extinction (Valiente-Banuet et al., 2006).

Acknowledgements

We thank A. Montesinos for comments on the first drafts of this manuscript and for many stimulating discussions on the role of mycorrhizae in plant–plant interactions. To P. Edwards and the reviewers for valuable comments to the ms that improved it significantly. J. Hadfield helped with the MCMCglmm program. C. Silva and J. P. Castillo helped during field and laboratory work. Funding was provided by PAPIIT-DGAPA, UNAM (IN-202811-3; IN-213414-3), MICINN (CGL2011-29585-C02-01) and CYTED (Acción 409AC0369). This paper constitutes a partial fulfillment of the Graduate Program in Biomedical Sciences of the National Autonomous University of México (UNAM). L.S. acknowledges the scholarship and financial support provided by the National Council of Science and Technology (CONACyT), and UNAM.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ppees.2014.07.001.

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Supplementary Material

Nurses experience reciprocal fitness benefits from their distantly related facilitated plants.

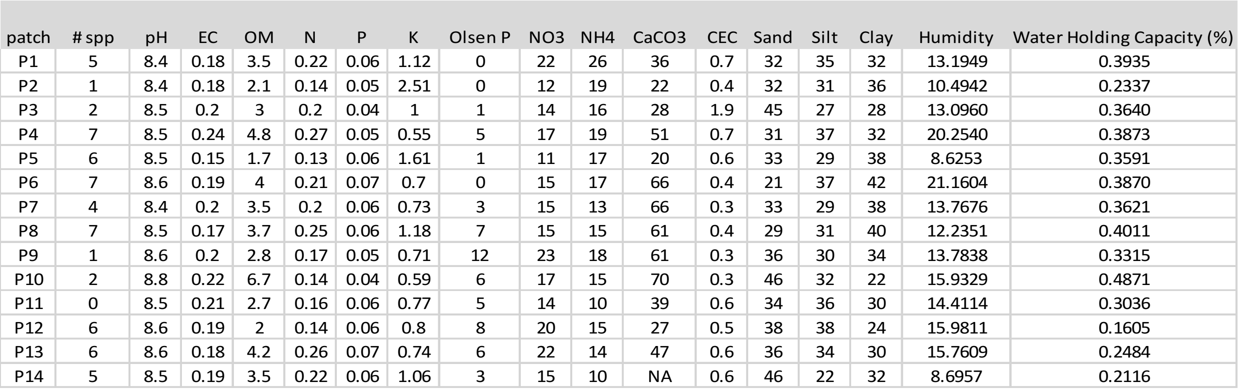
Table S1. Average ± SE physical and chemical soil variables underneath 20 *M. luisana* seedlings and neighboring gaps. Statistical t values given. No significant differences at α=0.05 were found between microsites in any variables measured (NS). Organic matter, N Total, Sand, Clay and Silt were arcsine transformed before analyses to improve normality.

Source	Gap (n=20)	*M. luisana* (n=20)	Paired t-test	Significance
Ca (meq/100g)	38.05 ± 1.05	36.39 ± 1.62	0.86	NS
K (meq/100g)	0.49 ± 0.05	0.43 ± 0.03	1.03	NS
Organic matter (%)	2.81 ± 0.22	3.39 ± 0.32	-1.47	NS
Mg (meq/100g)	1.99 ± 0.12	1.94 ± 0.19	0.18	NS
N Total (%)	0.14 ± 0.01	0.16 ± 0.01	-1.45	NS
N-NO_3^- (ppm)	15.85 ± 3.17	19.35 ± 2.52	-0.86	NS
N-NH_4^+ (ppm)	8.8 ± 0.62	7.7 ± 0.48	1.39	NS
Na (meq/100g)	0.09 ± 0.03	0.11 ± 0.06	0.77	NS
P (ppm)	12.55 ± 2.8	11.95 ± 1.6	0.18	NS
Clay (%)	42.2 ± 1.73	42.85 ± 1.89	-0.26	NS
Sand (%)	25.9 ± 2.56	21.6 ± 1.43	1.49	NS
Silt (%)	31.9 ± 7.13	35.55 ± 1.56	-1.84	NS

Table S2. Correlations of environmental variables (see raw values in the table below) with number of facilitated species in the patch.

Variables	r	p values adjusted for multiple tests (FDR method)
pH	-0.02	0.94
Electrical Conductivity (dSm^{-1})	-0.26	0.87
Organic Matter (%)	0.12	0.87
Total N (%)	0.55	0.33
Total P (%)	0.56	0.33
Total K (%)	-0.19	0.87
Olsen P(ppm)	-0.11	0.87
$N\text{-}NO_3^-$ (ppm)	0.09	0.87
$N\text{-}NH4^+$ (%)	0.15	0.87
$CaCO_3$ (%)	0.12	0.87
Cation Exchange Capacity (Cmol+ Kg^{-1})	-0.11	0.87
Sand (%)	-0.41	0.80
Silt (%)	0.22	0.87
Clay (%)	0.33	0.87
Gravimetric Humidity (%)	0.27	0.87
Water Holding Capacity (%)	0.02	0.94

Table S3. Raw values of microenvironmental soil parameters in different vegetation patches containing a variable number of facilitated species (# spp).



patch	# spp	pH	EC	OM	N	P	K	Olsen P	NO3	NH4	CaCO3	CEC	Sand	Silt	Clay	Humidity	Water Holding Capacity (%)
P1	5	8.4	0.18	3.5	0.22	0.06	1.12	0	22	26	36	0.7	32	35	32	13.1949	0.3935
P2	1	8.4	0.18	2.1	0.14	0.05	2.51	0	12	19	22	0.4	32	31	36	10.4942	0.2337
P3	2	8.5	0.2	3	0.2	0.04	1	1	14	16	28	1.9	45	27	28	13.0960	0.3640
P4	7	8.5	0.24	4.8	0.27	0.05	0.55	5	17	19	51	0.7	31	37	32	20.2540	0.3873
P5	6	8.5	0.15	1.7	0.13	0.06	1.61	1	11	17	20	0.6	33	29	38	8.6253	0.3591
P6	7	8.6	0.19	4	0.21	0.07	0.7	0	15	17	66	0.4	21	37	42	21.1604	0.3870
P7	4	8.4	0.2	3.5	0.2	0.06	0.73	3	15	13	66	0.3	33	29	38	13.7676	0.3621
P8	7	8.5	0.17	3.7	0.25	0.06	1.18	7	15	15	61	0.4	29	31	40	12.2351	0.4011
P9	1	8.6	0.2	2.8	0.17	0.05	0.71	12	23	18	61	0.3	36	30	34	13.7838	0.3315
P10	2	8.8	0.22	6.7	0.14	0.04	0.59	6	17	15	70	0.3	46	32	22	15.9329	0.4871
P11	0	8.5	0.21	2.7	0.16	0.06	0.77	5	14	10	39	0.6	34	36	30	14.4114	0.3036
P12	6	8.6	0.19	2	0.14	0.06	0.8	8	20	15	27	0.5	38	38	24	15.9811	0.1605
P13	6	8.6	0.18	4.2	0.26	0.07	0.74	6	22	14	47	0.6	36	34	30	15.7609	0.2484
P14	5	8.5	0.19	3.5	0.22	0.06	1.06	3	15	10	NA	0.6	46	22	32	8.6957	0.2116

Fig. S1. *Mimosa luisana* growing alone (A) and a phylogenetically diverse vegetation patch (B) formed by *M. luisana* shrub (the pink-white flowered plant) facilitating *Neobuxbaumia tetetzo*, *Siphonoglossa ramosa*, and *Mammillaria colina.* Photograph by Lugui Sortibrán.

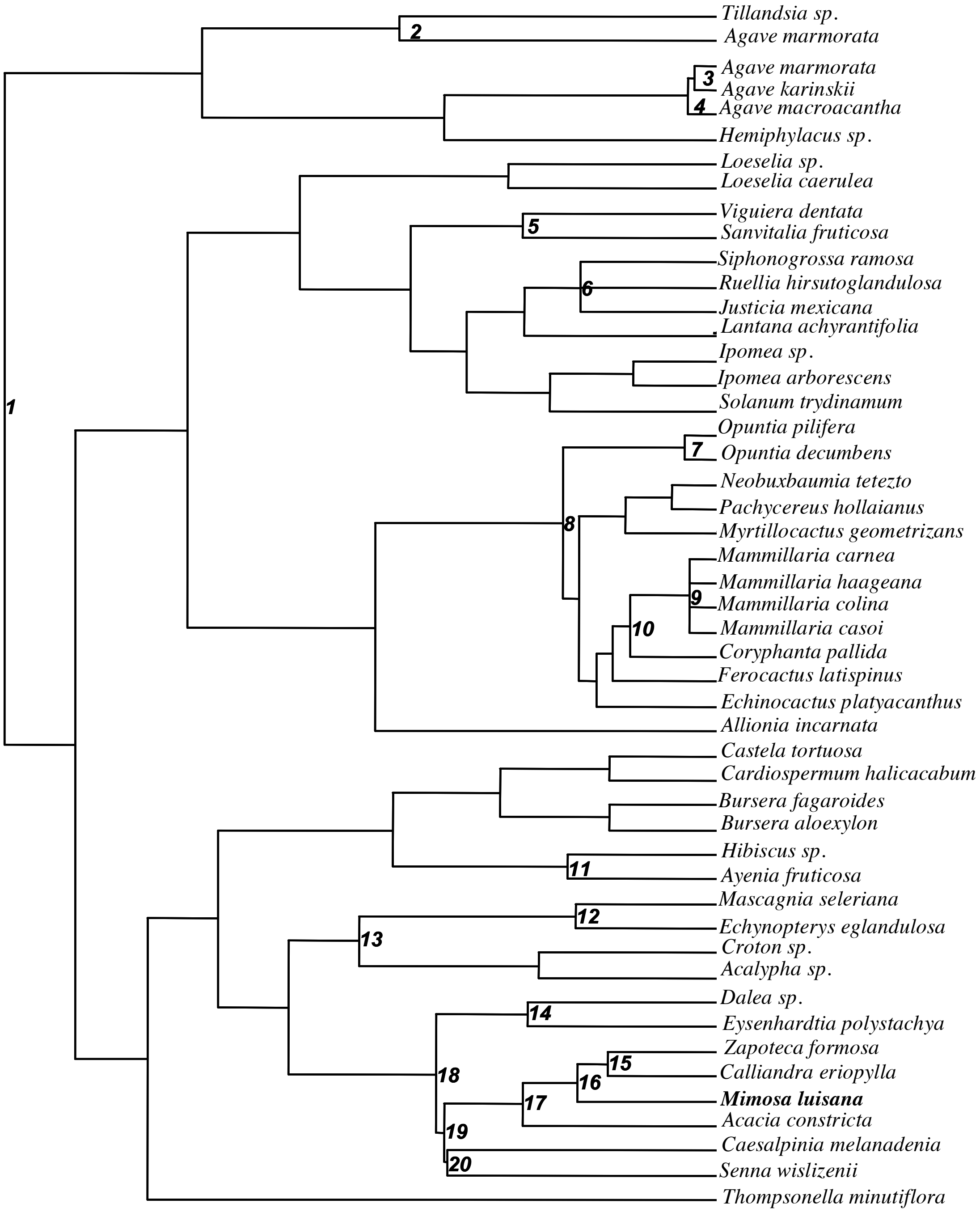


Fig. S2. Final phylogenetic tree fully resolved with the exception of two polytomies. Node label indicates the nodes with a fixed age used to calibrate the tree. Age estimates were obtained from Wikstrom *et al*. (2001, nodes 1, 2, 5-6, 11-13); Good-Avila *et al*. (2006, nodes 3-4); Arakaki *et*. (2011, nodes 7-10); and Simon *et al*. (2009, nodes 14-20).

CAPÍTULO II

Nurse plants benefit from facilitative interactions through mycorrhizae

NURSE PLANTS BENEFIT FROM FACILITATIVE INTERACTIONS THROUGH MYCORRHIZA[1]

Lugui Sortibrán[2], Miguel Verdú[3] and Alfonso Valiente-Banuet* [2,4]

[2] Departamento de Ecología de la Biodiversidad, Instituto de Ecología, Universidad Nacional Autónoma de México. A. P. 70-275, C. P. 04510, México, Cd. México.
Telephone number: +52 -55- 56-22-90-10. E-mail: luguisortibran@gmail.com
[3] Centro de Investigaciones sobre Desertificación (CIDE, CSIC-UV-GV), Carretera Moncada - Náquera, Km. 4.5, 46113 Valencia, Spain. E-mail: Miguel.Verdu@uv.es
[4] Centro de Ciencias de la Complejidad, Ciudad Universitaria, Universidad Nacional Autónoma de México, 04510, D. F.
*Correspondence: E-mail: avalib@gmail.com

Running title: Mutualism mediated by mycorrhiza

PREMISE OF THE STUDY: Plant facilitation is a positive interaction that 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, with *Mimosa luisana* representing a keystone nurse for many of them. *M. luisana* individuals manifest greater fitness when growing in association with their facilitated plants, compared to individuals growing in isolation. This observation suggests that nurses also benefit from their facilitated plants, a benefit thought to be mediated by mycorrhizal fungi.

METHODS: Under field conditions, we experimentally test whether mycorrhizal fungi mediate the increased fitness that the nurse *M. luisana* experiences when growing in association with its facilitated plants. We applied fungicide to the soil in order to reduce the mycorrhizal colonization of roots and then assessed the effect on the quantity and quality of seed production, in order to measure the different fitness components of nurses growing isolated, as opposed to associated with their facilitated plants.

KEY RESULTS: Fungicide application reduced the total number of seeds, number of seeds per pod, seed mass and seed viability of *M. luisana,* when growing associated with their facilitated plants, but not when growing in isolation.

CONCLUSIONS: These results indicate that nurses are benefitting from the presence of their facilitated plants through mycorrhizae, indicating that both aboveground and belowground, mutualistic communities are all part of one system, coexisting by means of intrinsically linked interactions.

KEY WORDS: Aboveground-belowground interactions; facilitation; mutualism; mycorrhizal fungi; species coexistence.

Recognition that aboveground and belowground communities form major components of ecosystems interacting in ongoing feedback implies a major step forward in comprehensive understanding of species coexistence and vegetation dynamics (Wardle et al., 2004; Van der Putten, 2009; Van der Putten et al., 2013; Bardgett and Wardle, 2010). For example, plant diversity can be explained in terms of a process of soil community feedback, through which the density and composition of microbes are altered in response to the composition of plant community and subsequently, the change in microbial composition modifies the performance of plants (Bever et al., 2010).

Plant species may coexist, even though they occupy different niches or when their relative fitness is similar (Chesson, 2000). Plant facilitation is a positive interaction between species, which may differ in their regeneration niche: some nurse species are able to recruit under stressful conditions, whereas facilitated species need more benign conditions to recruit. In arid environments, plant coexistence is strongly influenced by facilitative interactions that are strongly 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 that are maintained over time, in multispecific vegetation patches (Valiente-Banuet and Verdú, 2008). These vegetation patches are ubiquitous in many ecosystems (Hacker and 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. There is contradictory evidence of reduced and increased fitness in the case of nurse species, as a consequence of facilitative interaction (Schöb et al., 2014a, 2014b, 2014c; Sortibrán et al., 2014), probably reflecting negative and positive plant-soil feedbacks (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 mycorrhizal may affect nurse fitness.

Positive effects of facilitated plants on nurses would explain the maintenance of facilitative interactions until adult stages in multispecific vegetation patches, effects which may be mediated by mycorrhiza (Verdú et al. 2010). Mycorrhiza can transport water, nutrients and chemical defenses from facilitated plants directly to their nurses (through common mycorrhizal networks; Newman, 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 plants through mycorrhizal networks has been extensively documented (Giovanneti et al., 2004; Egerton-Warburton et al., 2007; Mikkelsen et al., 2008; Simmard et al., 2012; Montesinos-Navarro et al., 2016) but the ecological significance for plant performance is still debated (Van der Heijden and 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, hyphal exudates of mycorrhyza from facilitated plants may alter bacterial communities and accelerate mineralization rates, increasing the availability of nutrients in soil (Zhang et al., 2016). All these mechanisms may explain how nurses can benefit from their facilitated plants through mycorrhiza. However, positive effects of mycorrhiza on plant coexistence may transform to negative at certain resource levels, where the nutrient demand from each plant is altered (Jiang et al 2017).

Here, we explore the role of AMF communities involving facilitative interactions between a keystone nurse, *Mimosa luisana*, and its facilitated plant species in a Mexican desert community governed by facilitation (Valiente-Banuet and Verdú, 2007, 2008; Verdú et al. 2010; Verdú and Valiente-Banuet 2011). In this community, *M. luisana* is the only species able to recruit on the open ground and therefore it acts as the primary nurse for most of the species (Verdú and Valiente-Banuet 2008). Interestingly, *M. luisana* individuals coexisting with their facilitated species have been shown to have greater fitness than isolated individuals (Sortibrán et al., 2014), although it remains unclear whether these benefits are mediated by AMF. *M. luisana* is the generalist species with the greatest number of AMF partners in the plant-arbuscular mycorrhizal fungal community (Montesinos-Navarro et al., 2012b). Likewise, there is evidence that AMF promote nitrogen 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 mycorrhiza. 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 different fitness components. Based on previously explained observations, we hypothesize that the reduction in mycorrhiza abundance will produce a decrease in fitness among nurses, growing in association with their facilitated plants.

MATERIALS AND METHODS

Study area and species —This study was conducted in the semiarid Valley of Zapotitlán (18º 20´N, 97º 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 21º C with rare frosts (García, 1988). Specifically, the study site is located within the Botanical Garden

“Helia Bravo Hollis”, a natural unmodified protected area, located approximately 30 km south of Tehuacán city, where vegetation comprises a xeric shrubland. This vegetation is dominated by the columnar cactus *Neobuxbaumia tetetzo* and different shrub species such as *Mimosa luisana, Mascagnia parviflora, Ipomoea arborescens, Aeschynomene compacta, Caesalpinia melanadenia, Calliandra eriophylla, Zapoteca formosa, Senna wislizenii, Agave marmorata, A. macroacantha, Jatropha neopauciflora*, among other taxa (Valiente-Banuet et al., 2000). All these species, with the exception of *Mimosa luisana,* recruit through facilitation. *M. luisana* is able to facilitate 48 out of 56 species recorded for this community (Verdú and Valiente-Banuet, 2008), forming discrete vegetation multispecific patches of up to 12 species and 42 individuals. Certain isolated *M. luisana* individuals can also be found separated from other vegetation patches by 1.5-2 meters (see Appendix S1 in Supplemental Data with the online version of this article). The area of the vegetation patches ranges from 1 to 10 m^2 and all facilitated individuals are located beneath the vertical projection of the *M. luisana* canopy. Previous work at the study site has characterized the interactions between plants and Arbuscular Mycorrhizal Fungi (AMF) as a bipartite network characterized by a non-random, nested topology, where *M. luisana* acts as a hub connected to many AMF nodes (Montesinos-Navarro et al., 2012a). Moreover, up to 165 ± 16 AMF spores have been reported under the canopy of *M. luisana* (in 100 g dry weight of soil) mainly pertaining to the *Glomus* and *Acaulospora* genus(Camargo-Ricalde and Dhillion, 2003).

Mimosa luisana is a deciduous spiny shrub reaching heights of up to 2.2 m. Reproduction initiates when plants reach 0.80 m tall. Pink flowers which grow in spike infloresences 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 2 to 8 seeds; pods are grouped into bunches of 2 to 10 (Grether et al., 2006).

***Field experiment*—**Sampled vegetation patches were distributed within an area of ca. 750 m^2 (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 colonized 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). Nutrient conditions in plots with bare soils and plots where seedlings of *M. luisana* have become established do not differ statistically, indicating that this nurse 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 alone or associated with neighbors. The experimental design followed a 2 × 2 factorial design, crossing fungicide treatment (fungicide vs. control) with growing condition (alone vs. 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), a contact fungicide with non-systemic effects, very efficient for eliminating mycorrhizae without affecting soil, insects, bacteria or plant growth (Gange et al., 1990; Ganade and Brown, 1997; Hernández-Dorrego and Mestre-Parés, 2010). Despite the specificity of fungicide on mycorrhyza, the possibility of a reduction in the abundance of soil-borne fungal pathogens could not be dismissed. If this were the case, contrary to our hypothesis, this would indicate that fungicide benefitted nurse plants. Similarly, collateral effects resulting from fungicide like fertilization, due to its chemical composition or to the decomposition of dead organisms, would also imply that results contradict our hypothesis. The fungicide was applied at the rate of 2.0 g L^{-1} water (Ganade and Brown, 1997) at intervals of 3 weeks, prior to the rainy season (February-July) and six times over two years (2011 and 2012) to avert fungicide runoff with rain. In the control treatment, the same amount of water was added, as to the fungicide treatments. Fungicide and water were administered 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 m^2, throughout the entire soil surface below the canopy of *M. luisana*.

Fungicide efficacy on AMF colonization—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 twenty fragments 2 cm in length, per plant. We estimated the reduction in the percentage of root colonization of AMF, following the Phillips and Hayman method (1970) with slight modifications as follows: root samples were stored in 50% alcohol in September 2012. In the laboratory, roots were clarified in 10% aqueous solution of KOH (w^{-v}) for 10 min under pressure at 120º C, washed with 10% H_2O_2 and acidified with 1% HCl (v^{-v}). Subsequently, roots were stained with 0.05% Trypan blue (w^{-v}) in lactoglycerol. The percentage of root length colonized by AMF was estimated by a modified line intersection method (McGonigle et al., 1990) by placing the 20 root samples on slides and examined at 400× magnification, using an optical light microscope B201, Olympus, 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 *Mimosa 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 5 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 and germinated to assess seed viability. Seeds were scarified with sandpaper, prior to germination tests that were performed in Petri dishes with wet filter paper, at 25ºC in 12 h light/dark. 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. Nurse size was estimated by assuming that *M. luisana* is an inverted cone with an elliptical base, and calculating volume after measuring the height and two perpendicular diameters of the canopy cover.

***Statistical analysis*—**We compared the crossed effects of treatment (control *vs*. fungicide) and the growing condition of *M. luisana* (alone *vs*. associated) on AMF root colonization and on different components of reproductive output of *M. luisana,* applying Generalized Linear Models (GLM) with different error structures, depending on the nature of the dependent variable. For variables recording presence *vs.* absence (i.e., hyphae or vesicles in roots observed in 100 fields under the light microscope) or success vs failure (i.e., seed germination), we used a binomial distribution of errors. To account for overdispersion of the model, we used the quasibinomial family in the GLM. For calculations, such as total number of seeds or number of seeds per pod, produced by *M. luisana* individuals, we used quasipoisson distribution of errors. 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 co-variable, but later removed because it did not prove significant in any case. All statistical analyses were performed with R software version 3.2.1 (R Development Core Team, 2015).

RESULTS

The fungicide significantly reduced AMF hyphae colonization 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 colonization 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 in terms of all four aspects (total number of seeds, seeds per pod, seed mass, and seed viability) as indicated by significant statistical interactions in 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). In all previous analyses, the size of the plant showed no significant effect, so it was removed from all models.

DISCUSSION

Our results mostly show that *M. luisana* nurses obtain fitness benefits from their facilitated adult plants through mycorrhizae, illustrating a mechanism by which a facilitative interaction may also be beneficial for the nurse and explaining how above 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 colonization of *M. luisana* roots was reduced, possibly diminishing 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 and Vadez, 2002; *Marschner, 2012; Hushof et al., 2012;* DiManno and Ostertag, 2016), our results strongly suggest that nurses living with their facilitated plants have greater access to resources *via* mycorrhiza. Fungicide may have also produced other effects, such as pathogenic fungi reduction and increased nutrient availability, due to decomposition of dead microorganisms (Allison et al., 2007; Berns et al., 2008; Goberna et al., 2011). However, if these effects had been quantitatively important, the fungicide treatment should have produced a benefit for *M. luisana* in terms of increased seed production and/or seed quality. In contrast, 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 (Valiente-Banuet and Verdú, 2008) and harbors the highest diversity of AMF (Montesinos-Navarro et al., 2012b). Plant facilitation is stronger among those species that differ in their AMF, indicating a clear relationship between plant facilitation and mycorrhizae (Montesinos-Navarro et al., 2012a). *M. luisana,* by facilitating other species increase the abundance and diversity of fungi in the rhizosphere and may benefit directly or indirectly from the complementary functions provided by different fungi. Experimental evidence indicates that plants colonized with multiple AMF species obtain more phosphorus and grow more than those colonized with only one species (Van der Heijden et al, 1998; Johnson et al., 2004; Jansa et al., 2008). A possible mechanism explaining why enriched rhizospheres benefit plants, is the transfer of nutrients between plants (Garg and 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 (Smith and Read, 1998; Leake et al., 2004; Selosse et al., 2006; Bucher, 2007; Simard et al., 2012; Yang et al., 2013). Mycorrhiza may also redistribute water in the soil, increasing the rate of diffusion and the absorption of nutrients (Eguerton-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 15N stable isotopes, Montesinos-Navarro et al. (2016) showed that soil fungi promote nitrogen transfer from *M. luisana* to its facilitated species.

Another experimental work used deuterium-labelled water to show that facilitated plants may transfer water to *Mimosa luisana* (Montesinos-Navarro et al., unpublished). Similarly, it is possible that other nutrients are transferred between neighboring plants via mycorrhiza such as that reported elsewhere (Selosse et al., 2006; Van de Heijden and Horton, 2009; Mikkelsen et al., 2008; Garg and Chandel, 2010; Simard et al., 2012; Yang et al., 2013). 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 mycorrhiza 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 extraradical mycelia in *M. luisana* individuals associated with their facilitated plants, as a result of multiple nutrients provided to fungi by all the facilitated plant species. More extensive extraradical 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 be not as extensive. This difference in the size of extra-radical mycelia between nurses, growing in association or isolation, may explain why fungicide reduced the fitness of the former, but not that of the latter. Similarly, an extensive mycorrhizal system 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 mycorrhiza can also participate (Prieto et al. 2012).

Unfortunately, using our data, it is impossible to separate the relative contributions made by all these, non-exclusive mechanisms; however, an unequivocal pattern of nurse benefits, mediated by mycorrhiza is revealed that deserves further research. Contrary to traditional thinking, which suggests that competition is the main mechanism assembling ecological communities, our results show that above- and belowground mutualistic communities are all part of one system, whose positive interactions ultimately determine coexistence.

ACKNOWLEDGEMENTS

We thank R. Cruz-Ortega, A. Montesinos-Navarro and P. Vinuesa for valuable comments and for many stimulating discussions while preparing this ms. A. Arizmendi, C. Silva and J.P. Castillo helped during field and laboratory work. This work was supported by PAPIIT-DGAPA, UNAM (IN-213414-3; IN-210117), MICINN (CGL2014-58333-P) and CYTED (Acción 409AC0369) for funding the study. L. Sortibrán acknowledges the scholarship and financial support provided by the National Council of Science and Technology (CONACyT, 103137), and UNAM. This work is presented in partial fulfillment towards L. Sortibrán doctoral degree in the Programa de Doctorado en Ciencias Biomédicas de la Universidad Nacional Autónoma de México (UNAM).

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TABLE 1. Effects of the fungicide treatment (control *vs* fungicide) on AMF root colonization and reproductive output of *M. luisana* individuals growing under different conditions (isolated *vs*. associated). Each column shows the estimates and SE of the corresponding Generalized Linear Model.

	% Root colonization		Reproductive output			
	Hyphae	Vesicle	Seed number	Seeds/pod	Seed mass	Seed viability
Condition	0.26±0.26	0.41 ± 0.28	2.23 ± 0.86*	0.23 ± 0.05*	0.47 ± 0.004*	1.82 ± 0.56*
Treatment	-2.12 ± 0.29*	-1.31 ± 0.41*	0.90 ± 0.99	0.06 ± 0.05	-0.04 ± 0.05	-0.03 ± 0.65
Condition x Fungicide	-0.73 ±0.38ms	-1.25 ± 0.56*	-1.78 ± 1.00 ms	-0.02 ± 0.04*	-0.47 ± 0.06*	-1.65 ± 0.77*

*$p<0.05$; ms $0.05<p<0.10$

Figure Legend

FIGURE 1 Fungicide effects on A) percent root length colonized by hyphae; B) percent root length colonized by vesicles; C) total seed production; D) seeds per pod; E) seed mass and F) seed germination percentage of *Mimosa luisana* individuals growing alone and associated with their facilitated plants in control and fungicide treatments. Values are mean ± SE. Asterisks indicate significant differences for the effect of fungicide within each condition at 0.05 level.

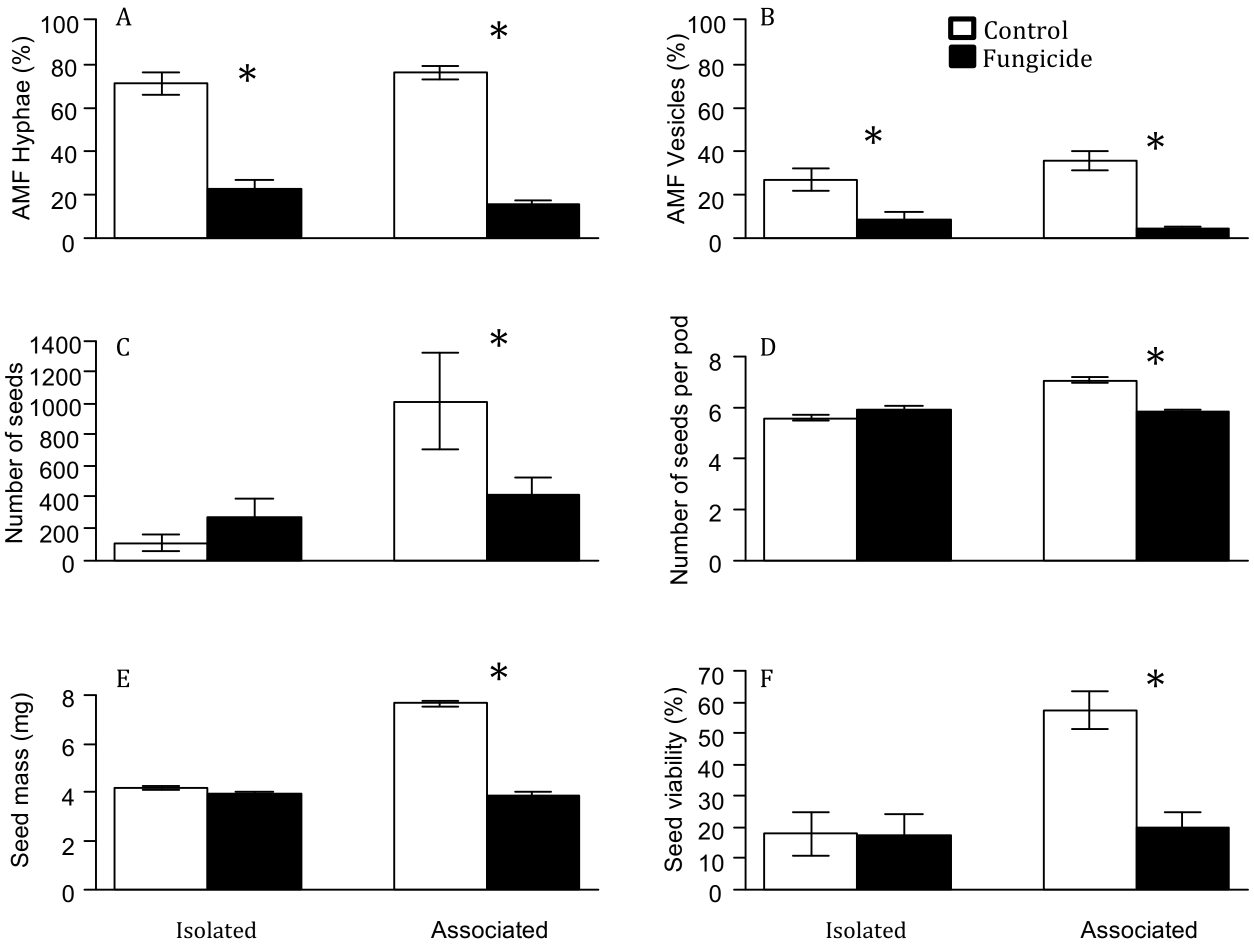

A
AMF Hyphae (%)
100
80
60
40
20
0
*
*
B
AMF Vesicles (%)
Control
Fungicide
*
*
C
Number of seeds
1400
1200
1000
800
600
400
200
0
*
D
Number of seeds per pod
8
6
4
2
0
*
E
Seed mass (mg)
*
F
Seed viability (%)
70
60
50
40
30
20
10
0
*
Isolated
Associated
Isolated
Associated

Fig. 1

Supplementary Material

Nurse plants benefit from facilitative interactions through mycorrhizae

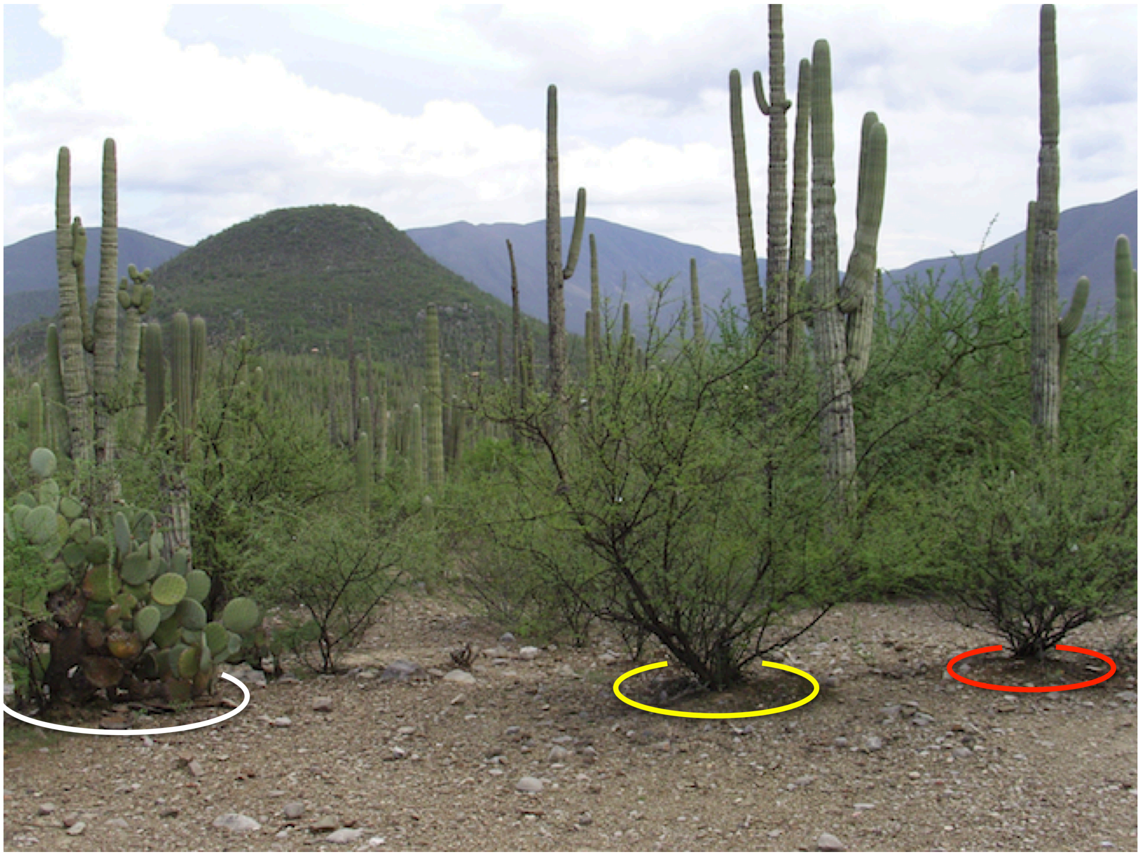


Fig. 1 Study site showing three vegetation patches in which *M. luisana* grows associated multispecifically with the facilitated plants *Neobuxbaumia tetezo*, *Opuntia pilifera, Thompsonella minutiflora*, *Mammillaria colina Coryphantha pallida* and *Siphonoglossa ramosa* (white circle); growing associated to *T. minutiflora* (yellow circle); and growing in isolation (red circle). The distance between patches ranges from 1.5 to 2.5m. Photograph by Lugui Sortibrán.

CAPÍTULO III

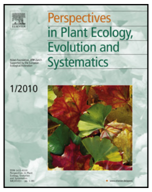
Soil fungi promote nitrogen transfer among plants involved in long-lasting facilitative interactions

Perspectives in Plant Ecology, Evolution and Systematics 18 (2016) 45–51

Contents lists available at ScienceDirect

Perspectives in Plant Ecology, Evolution and Systematics

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



Soil fungi promote nitrogen transfer among plants involved in long-lasting facilitative interactions

Alicia Montesinos-Navarro[a,b,*], Miguel Verdú[b], José Ignacio Querejeta[c], Lugui Sortibrán[a], Alfonso Valiente-Banuet[a,d]

[a] Departamento de Ecología de la Biodiversidad, Instituto de Ecología, Universidad Nacional Autónoma de México, A. P. 70-275, C. P. 04510 México, D. F., Mexico
[b] Centro de Investigaciones sobre Desertificación (CIDE, CSIC-UV-GV), Carretera de Moncada-Náquera Km 4.5, 46113 Moncada, Valencia, Spain
[c] Departamento de Conservación de Suelos y Aguas, Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC), Campus de Espinardo, PO Box 4195, E-30100 Murcia, Spain
[d] Centro de Ciencias de la Complejidad, Ciudad Universitaria, Universidad Nacional, Autónoma de México 04510, D. F., Mexico

ARTICLE INFO

Article history:
Received 15 July 2015
Received in revised form
26 November 2015
Accepted 8 January 2016
Available online 11 January 2016

Keywords:
Adult-facilitated benefits
Common mycorrhizal network
Community assembly
Mimosa luisana
^{15}N transfer

ABSTRACT

Plant facilitative interactions may persist in the long term when there are benefits for the interacting adult plants. Whereas persistent benefits for adult nurse plants have been demonstrated, the long-term benefits derived by adult facilitated plants have been largely unexplored. We hypothesize that common mycorrhizal networks (CMNs) can provide a pathway through which nurse species can benefit adult facilitated plants persistently. We specifically test whether nitrogen can be transferred from nurse plants to their adult facilitated plants, and evaluate to which extent CMNs mediate the transfer. We selected 32 adult individuals of 6 facilitated plant species growing in 15 vegetation patches in a Mexican desert. We treated some vegetation patches with fungicide and left others as controls. Then, we labeled the nurse plants with ^{15}N-enriched urea and quantified the amount of ^{15}N transferred to their adult facilitated plants. We expected a greater ^{15}N transfer to facilitated individuals growing in vegetation patches with intact CMNs than in those treated with fungicide. Facilitated plants growing in patches with intact CMNs showed on average a greater increment in their foliar δ^{15}N (i.e. difference between post-labeling–pre-labeling) than those in patches treated with fungicide. Our results provide evidence that CMNs enhance nitrogen transfer among adult plants, thus providing a potential mechanism contributing to the long-term persistence of plant facilitative associations.

1. Introduction

The ecological mechanisms underlying plant facilitative interactions are well known, but a deep understanding of their evolutionary implications is still lacking (Brooker et al., 2008, Valiente-Banuet and Verdú, 2013). In order to explore the effects of facilitative interactions in the fitness of the interacting plant species, a first step is to assess whether the benefits resulting from the interaction are maintained along the ontogeny of the interacting plants. In facilitative interactions, at least one of the associated species gets a benefit (facilitated species) without resulting in any damage for the other (nurse species) or even providing a benefit to it (Callaway, 2007; Sortibrán et al., 2014). Benefits for adult nurse plant have been reported in arid environments, with nurse plants producing more flowers and fruits when growing associated with their facilitated plants than when growing alone (Sortibrán et al., 2014, unpublished results). However, in alpine systems, the nurse plant benefits can shift to costs depending on the reproductive trait measured (Schöb et al., 2014). In addition, while adult nurse plants facilitate the establishment of seedlings of facilitated species, asymmetric competition later on in the ontogeny of the facilitated plants can result in mortality of the adult facilitated plants (Valiente-Banuet and Verdú, 2008; Armas and Pugnaire, 2009; Rolo et al., 2013). However, other facilitative interactions persist over time resulting in adult nurse and facilitated plants associations (Valiente-Banuet and Verdú, 2008). In persistent interactions which can reach 57% of all the facilitative interactions (Valiente-Banuet and Verdú, 2008), the adult facilitated plants may still receive some benefits from being associated with their nurse plant. The mechanisms by which nurse plants can

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* Corresponding author.
E-mail addresses: ali.montesinos@gmail.com (A. Montesinos-Navarro), Miguel.Verdu@uv.es (M. Verdú), querejeta@cebas.csic.es (J.I. Querejeta), luguisortibran@gmail.com (L. Sortibrán), avalib@gmail.com (A. Valiente-Banuet).

http://dx.doi.org/10.1016/j.ppees.2016.01.004
1433-8319/

promote facilitated seedlings establishment have received considerable attention (Nara and Hogetsu, 2004; Nara, 2006; Richard et al., 2009; Teste et al., 2009; Van der Heijden and Horton, 2009; Booth and Hoeksema, 2010; Bingham and Simard, 2011, 2012; Molina-Montenegro et al., 2015). However, the mechanisms enhancing the benefits for adult facilitated plants have been largely unexplored.

Mycorrhizal fungi may play an important role in the persistence of plant facilitative interactions, considering their influence in the outcome of plant–plant interactions in a wide variety of ecosystems, including alpine, Mediterranean, marshland, dune shore, forest, prairies and deserts (Hartnett et al., 1993; Nara and Hogetsu, 2004; Nara, 2006; Richard et al., 2009; Booth and Hoeksema, 2010; Grau et al., 2010; Casanova-Katny et al., 2011; Montesinos-Navarro et al., 2012a; Zhang et al., 2014; Molina-Montenegro et al., 2015). The mycelia of mycorrhizal fungi can colonize the roots of neighbor plants, establishing common mycorrhizal networks (CMN) that allow intra and interspecific transference of resources between plants (Newman, 1988; Simard and Durall, 2004; Selosse et al., 2006). Plants connected through CMN can exchange signals promoting genes involved in defense against pathogen infection (Song et al., 2010), induce the production of volatiles to protect neighbor plants from herbivores (Babikova et al., 2013), or transfer allelochemicals which expands the action range of these regulators of plant competition (Barto et al., 2011). Resource translocation though CMN promotes seedling growth and survival through water and nitrogen transfer from adult donors (Teste et al., 2009; Booth and Hoeksema, 2010; Bingham and Simard, 2011), which in the long term can give rise to emergent patterns at the plant community level. Mycorrhizal fungi can affect plant communities by reducing interspecific competition among co-existing plant species when the diversity of mycorrhizal fungi increases (Wagg et al., 2011). Furthermore, the assembly of plant and mycorrhizal communities seems to be closely interrelated as suggested by a correspondence between the phylogenetic structures of mycorrhizal and plant communities in vegetation patches (Montesinos-Navarro et al., 2015). Both mycorrhizal and plant assemblages can in turn influence each other a follows. On the one hand, mycorrhizal species richness can promote plant diversity and productivity (Van der Heijden et al., 1998; Vogelsang et al., 2006; Maherali and Klironomos, 2007). On the other hand, plant facilitative interactions can indirectly influence mycorrhizal assemblages, as nurses tend to associate with facilitated species that increase the mycorrhizal richness in the shared rhizosphere (Montesinos-Navarro et al., 2012a). Therefore, the outcome of facilitative interactions can be mediated by the mycorrhizal fungi shared in the rhizosphere, and the mycorrhizal community can be in turn shaped by the plant species involved in the facilitative interactions. In this sense, the nurse plant performance is enhanced when surrounded by a rich and phylogenetically diverse neighborhood of facilitated plant species (Brooker et al., 2008; Sortibrán et al., 2014), what can be partially influenced by the presence of CMNs in the soil (Sortibrán et al., unpublished results). Despite the potential of CMNs to influence the persistence of facilitative interactions, the specific mechanisms by which CMNs can promote facilitative interactions between adult plants are largely unknown.

Inter-connected plants can exchange water and nutrients along source-sink gradients (Bethlenfalvay et al., 1991; Frey and Schuepp, 1992; Simard et al., 1997, 2012; Egerton-Warburton et al., 2007; Querejeta et al., 2012). In the case of nitrogen, natural nitrogen (N), source-sink gradients can result from the association of legume and non-legume species, as legumes in symbiosis with N_2-fixing bacteria have access to atmospheric N, inaccessible to other plants (Dilworth et al., 2008). It is well known that legumes play an important role in structuring plant communities through plant facilitative interactions (Barnes and Archer, 1996; Flores and Jurado, 2003; Liphadzi and Reinhardt, 2006), and the N-transfer from a nurse legume to facilitated plants could be an ecologically relevant mechanism influencing plant facilitative interactions. The nitrogen transfer from legumes to non-legumes mediated by CMN has been largely studied sowing crop species in managed agroecosystems (Hamel et al., 1991; Hamel and Smith, 1991, 1992; Frey and Schuepp, 1992; Johansen and Jensen, 1996; He et al., 2004, 2005, 2006; Wichern et al., 2007; Teste et al., 2009; Laberge et al., 2011; Rasmussen et al., 2013; Chalk et al., 2014). However, far less research has been conducted in natural communities (but see He et al., 2006), and thus little is known about the role of nitrogen transfer in more complex systems where multiple species can interact.

In this paper, we propose to test whether CMNs can promote nitrogen transfer between adult plants involved in long-lasting plant facilitative interactions. We selected an arid system in which we had previous experimental evidence that vegetation patches were originated by plant–plant facilitation processes (Castillo et al., 2010). We focus on species that were initially facilitated by a nurse species (i.e. growing within the same vegetation patch) and have survived until their adult stage (long-lasting facilitation interactions). We hypothesized that CMNs mediate N transfer from the nurse to adult facilitated plants. We selected 32 adult individuals of 6 facilitated species growing in 15 vegetation patches resulting from the facilitation process triggered by the legume shrub *Mimosa luisana*. Following a balanced design, we treated a group of vegetation patches with fungicide, and another control group with water. Afterwards, we labeled the nurse plants with a ^{15}N-tracer, and quantified the ^{15}N transfer from the nurse plants to their facilitated plants. We expected greater N transfer to the facilitated plants in vegetation patches with intact CMNs (control), compared to individuals in vegetation patches treated with fungicide, and suggest that this can be a potential mechanism contributing to the persistence of long-lasting plant facilitative interactions.

2. Materials and methods

2.1. Study area

This experiment was conducted in the semiarid Valley of Zapotitlán (18°21′ N, 97°28′ W), a local basin of the biosphere reserve of Tehuacán-Cuicatlán Valley in the state of Puebla, Mexico. Aridity in this region is due 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 (June–August), and an annual mean temperature of 21 °C with rare frosts (García, 1988). Specifically, the study site is located 30 km south of Tehuacán city in a xeric shrubland dominated by the columnar cactus *Neobuxbaumia tetetzo*, and shrub species such as *Mimosa luisana*, *Mascagnia seleriana*, *Ipomoea arborescens*, *Aeschynomene compacta*, *Caesalpinia melanadenia*, *Calliandra eryophylla*, *Zapoteca formosa*, *Senna wislizenii*, *Agave marmorata*, *Agave macroacantha* and *Jatropha neopauciflora* (Valiente-Banuet et al., 2000).

2.2. Plant–plant facilitation measurement

To verify that current community structure was governed by facilitation, in 2007 the cover of perennial plants and bare ground was measured in four 1000 m^2 plots. For each species, the number of seedlings and saplings (<30 cm height) growing beneath plant canopies and in bare ground areas was counted. Then, a contingency analysis was conducted for all species together to compare the number of young individuals growing beneath nurse plant canopies vs. bare ground (Table 1). Plant facilitative interactions were confirmed to be driving the community structure resulting in

Table 1
Regeneration niche of species in the community. Species are considered facilitated if the χ^2-test is significant, and the observed number of individuals (all species pooled) recruiting under nurses is higher than expected by chance.

Number of species	Number of nurse species	% species facilitated	Number of ind. in open space	Number of ind. under nurse species	Total plant cover (%)	Bare ground cover (%)	χ^2-value	*P*-value
56	21	96	92	1237	71.1	28.9	367.5	<0.00001

Adapted from Valiente-Banuet and Verdú (2007).

a significant greater amount of individuals recruiting under nurse plants than in the bare ground (Table 1).

In this system, the legume *M. luisana* is a key nurse plant for most of the species in the community, as 48 out of 56 of the species recorded recruit more frequently beneath it than expected by chance. These include species of several functional groups – shrubs, succulent plants such as *Agave* and cacti, perennial climbing vines, and perennial herbs (Valiente-Banuet and Verdú, 2007). Most importantly, *M. luisana* is the only nurse that can recruit in the bare ground and therefore it is responsible of the initial formation of a vegetation patch (Sortibrán et al., 2014).

2.3. *Vegetation patches as a proxy for plant–plant facilitation*

The inability of most species to recruit in the open ground resulted in a patchy environment in which vegetation is clumped under the canopy of the nurse plants, usually *M. luisana*. Therefore, species growing within a patch (i.e. under the canopy of an adult individual of *M. luisana*) can be considered to be species facilitated by this nurse species. In addition, it was considered a long-lasting facilitation interaction when an adult facilitated and nurse plants persist within the same patch. The area occupied by a vegetation patch ranged from 1 to 5 m^2, which corresponds to the vertical projection of the canopy of the nurse plant.

2.4. *Field experiment*

As the N isotopic composition of plant material depends on the age and type of tissue sampled (Dawson et al., 2002), we used species with leaves, which are produced in a relatively short amount of time. This constraint excluded cacti and agaves from the experimental design. We selected 32 adult individuals of 6 facilitated species growing in 15 vegetation patches with an adult *M. luisana*. The six selected facilitated species and the nurse species are known to host arbuscular mycorrhizal fungi (Montesinos-Navarro et al., 2012b). Individuals were distributed in vegetation patches following a balanced design, so that we could treat the same number of individuals per species with water (8 control patches) and the other half with fungicide (7 treated patches) (Table 2). These 15 vegetation patches were distributed within an area of 675 m^2, with control and treated patches interspersed in space and at least 5–10 m apart from the nearest patch. These patches were used in a previous 2-year experiment using the fungicide Rovral 50% (Iprodione), which eliminates fungi very effectively, especially arbuscular mycorrhizal fungi, without affecting soil insects and bacteria (Gange et al., 1990; Ganade and Brown, 1997; Hernández-Dorrego and Mestre-Parés, 2010). During our previous experiment, Rovral reduced the percentage of root colonization by mycorrhizal fungi in the roots of *M. luisana* from 73.8% to 22% (Sortibrán et al., unpublished results). All the 7 patches treated with fungicide in this experiment had been previously treated with the same fungicide during the two previous years (at the rate of 2.0 g/L of water, approx. 20 L per vegetation patch, at intervals of 3 weeks before the rainy season (six times) for 2 years), and 7 out of the 8 control patches had also been previously irrigated with the same amount of water. One more control patch was selected in 2013 to complete the balanced design, and similar results were observed in this patch compared to the other control patches. From May to July 2013 the fungicide and control treatments were restarted. Each 15 ± 5 days (four times), a dilution of 20–25 L of water with 2 g/L of the fungicide Rovral was applied in each of the treated patches and the same amount of water without fungicide was added to the control patches. During the application, the dilution was dispensed gradually using 3 or 4 canisters of 6 L, depending on the area of the vegetation patch. In order to prevent the leaching to other nearby vegetation patches, the dilution was dispensed into 3–5 holes, 5 cm deep each, dug in the ground of each patch. Considering that vegetation patches ranged from 1 to 5 m^2, this procedure ensured an even distribution of the fungicide throughout each patch.

2.5. *Nurse ^{15}N labeling*

At the beginning of August 2013, we labeled the nurse plants with urea enriched in ^{15}N following the methodology proposed by Putz et al. (2011). We prepared urea solution by dissolving 4 g of urea at 98% ^{15}N (Cambridge Isotope Laboratories, Inc.) in 2 L of

Table 2
Post-labeling foliar δ^{15}N value (‰) for each nurse and facilitated plant species in each vegetation patch (after 15 days of the application of the ^{15}N tracer to the nurse plants). Control patches are named as Ctr-1 to Ctr-8 and Fungicide-treated patches as Fung-1 to Fung-7.

Patch	Nurse	*Cathestecum brevifolium*	*Loeselia caerulea*	*Ruellia hirsutoglandulosa*	*Sanvitalia fruticosa*	*Siphonoglossa ramosa*	*Viguiera dentata*
Ctr-1	−0.19		4.27	2.59			
Ctr-2	45.85	2.76	3.48		2.97	1.26	
Ctr-3	109.09						3.4
Ctr-4	122.81	24.71			3.8	14.68	
Ctr-5	123.25				6.84		
Ctr-6	123.73	4.69		8.55			
Ctr-7	265.19				5.51		
Ctr-8	1162.33	2.8	6.85				
Fung-1	5.63	1.23	0.54		4.74	2.15	
Fung-2	14.47	0.53	2.07				
Fung-3	23.58					2.63	
Fung-4	28.93		0.58	3.1	−1.71		2.65
Fung-5	54.52	1.35			−0.32		
Fung-6	143.92				4.51		
Fung-7	1159.04	1.76		2.3			

water and 8 ml of surfactant. Each of the 15 *M. luisana* shrubs were systemically labeled by introducing individual branches in a 10 ml centrifuge tubes. In each *M. luisana* shrub, eight tubes were attached vertically to branches and sealed introducing the tube within a ziplock plastic bag and sealing the bag with tape to reduce evaporation and avoid spillage. A total of 80 ml of ^{15}N solution was provided to each *M. luisana* individual. Two weeks after the application, when most of the ^{15}N solution had been absorbed, the labeled branches were cut to remove the bag without spillage. Approximately 1 g of fresh leaves was collected from the 32 individuals of the facilitated species (16 in control and 16 in treated patches) right before and 15 days after the application of the ^{15}N labeling to the nurse plants (64 samples). Leaves were collected from different branches to represent the average foliar ^{15}N content in the whole canopy of the plant. In the case of nurse plants, we ensure that we avoid collecting the leaves where the ^{15}N solution was applied by cutting these branches as explained before.

2.6. *Sample preparation and stable isotope analysis*

Fresh leaves from all the facilitated and nurse plants (collected in the same individuals before and after the application of ^{15}N to the nurse plant) were dried at 50 °C for 3 days and then ground to a fine powder. We encapsulated 3 mg of plant material into tin capsules (8 × 5 mm Elementar Americas, Inc.) for nitrogen (δ^{15}N) isotope analysis. The University of California, Davis Stable Isotope Facility (SIF) conducted δ^{15}N isotope analyses using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 continuous flow isotope ratio mass spectrometer. SIF used conventional delta (δ) notation to report the relative difference of isotope ratios for samples (expressed in parts per thousand, ‰), and the international measurement standards N_2 atmospheric gas (air) (3.677×10^{-3}) for nitrogen (Coplen, 1994; IAEA, 2009). The precision of the δ^{15}N measurements was ±0.3 ‰.

2.7. *Data analysis*

In order to identify the significant sources of variation in foliar δ^{15}N in the facilitated plants, we used a Bayesian Generalized Linear Mixed Model. We tested for differences in foliar δ^{15}N as a function of "Time" (before (pre-labeling) vs. after (post-labeling) nurse ^{15}N-enrichment), "Treatment" (fungicide vs. control) and the interaction term (Time × Treatment) using orthogonal contrasts and Gaussian distribution of errors. We ran two independent models; one for the nurse species (*M. luisana*), to confirm the success of the labeling with ^{15}N, and another for the rest of species (facilitated species). In the latter case, we take into account that the δ^{15}N of the facilitated plants might not be independent if the samples were measured in: (a) the same individual, before and after the application of the ^{15}N to the nurse, (b) individuals of the same plant species, (c) plants growing in the same patch, (d) plants facilitated by a nurse with a given ^{15}N enrichment. To do so, we considered "facilitated individual plant", "facilitated species", "patch" and "nurse δ^{15}N value" as random factors in the model. The models were run with the help of MCMC techniques as implemented in the MCMCglmm package for R (Hadfield, 2010; R Development Core Team, 2011). We used the default priors and ran 2000000 MCMC iterations sampled each 1000 with a burn-in period of 25%. Convergence was assessed by visual inspection and it was checked that autocorrelation between successive stored iterations was lower than 0.1. The statistical significance of the factors in the model was estimated by calculating the 95% credible interval (CI) of their posterior distribution and checking afterwards that zero was not included in that interval. If CMN play a role in N transfer from the nurse plant to the facilitated species, we expect a significant interaction term between "Time" and "Treatment". Specifically, we expect a greater difference between the post-labeling–pre-labeling δ^{15}N (i.e. increment in δ^{15}N), in the facilitated plants growing in patches with intact CMNs (control patches) than in patches where CMNs had been reduced (patches treated with fungicide).

2.8. *Estimates of N transfer*

In order to quantify the average ^{15}N transfer between plants, only cases in which the nurse enrichment was unequivocal (i.e. nurse increment in $\delta^{15}\mathrm{N} > 10‰$) were selected. To estimate the percentage of ^{15}N tracer transferred from the nurse to the facilitated plant, sample δ^{15}N values were converted to absolute isotope ratio (R) as in Teste et al. (2009):

$$R_{\mathrm{sample}} = [(\delta^{15}\mathrm{N}/1000) + 1] \times R_{\mathrm{standard}}$$

The percentage contribution of the heavy isotope to the total number of atoms of that element in the sample (atom%) was calculated following Dawson et al. (2002):

$$\mathrm{atom\%}^{15}\mathrm{N} = 100 \times (R_{\mathrm{sample}}/(R_{\mathrm{sample}} + 1))$$

The background atom% values were subtracted from the sample values after the application of the ^{15}N to calculate the atom% excess (Teste et al., 2009):

$$\mathrm{atom\%}^{15}\mathrm{N\,excess} = \mathrm{atom\%}_{\mathrm{after}} - \mathrm{atom\%}_{\mathrm{before}}$$

Finally, following Tomm et al. (1994) the percentage of the ^{15}N tracer in the receiver derived from N transfer from the donor (% NDFT) was calculated as:

$$\%\,\mathrm{NDFT} = (\mathrm{atom\%}^{15}\mathrm{N\,excess}_{\mathrm{receiver}}/\mathrm{atom\%}^{15}\mathrm{N\,excess}_{\mathrm{donor}}) \times 100$$

3. Results

The ^{15}N labeling of the nurse plants was effective, as they significantly increased their foliar δ^{15}N values after 15 days of the tracer application, from 1.25 ± 0.30‰ to 225.48 ± 99.69‰ (mean ± SE); post mean = 111.59 [5.44, 213.54]. All the 15 nurse plants increased their foliar δ^{15}N after the application of the tracer, and all but two showed a δ^{15}N increment (i.e. difference between post-labeling–pre-labeling levels of δ^{15}N) greater than 10‰. One poorly ^{15}N-enriched nurse plant was in a control patch and the other in a fungicide-treated patch. There were no overall differences between the average foliar δ^{15}N values of nurse plants growing in control and fungicide-treated patches (post mean = −10.46 [−109.951, 97.03]), and nurse plants did not show a significant Time × Treatment interaction effect (post mean = −11.01 [−126.04, 80.60]).

However, considering facilitated species, there was a significant Time × Treatment interaction effect (Table 3). As expected, facilitated adult plants showed a greater increment (post-labeling–pre-labeling) in foliar δ^{15}N values in control patches than in patches treated with fungicide (Time × Treatment interaction

Table 3
Results of the Bayesian Generalized Linear Mixed Model explaining the variation in δ^{15}N in the facilitated plants as a function of Time (before vs. after nurse ^{15}N labeling), Treatment (fungicide vs. control) and the interaction between them. Statistically significant factors (*) in the model were those whose 95% credible interval of their posterior distribution did not include zero.

	Post mean	Lower CI-95%	Upper CI-95%
(Intercept)	3.36*	2.41	4.26
Time (before vs. after)	0.62	−0.19	1.49
Treatment (control vs. fungicide)	−1.35*	−2.27	−0.44
Time × Treatment	−0.85*	−1.73	−0.04

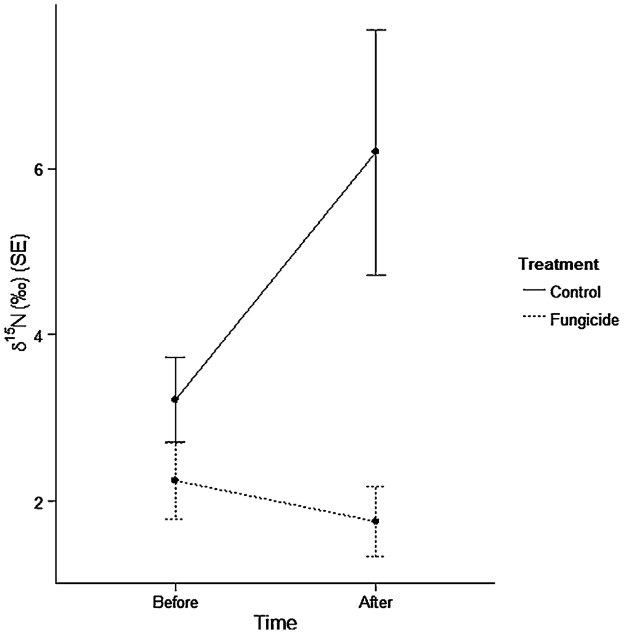

Fig. 1. Mean (standard error) foliar $\delta^{15}N$ of facilitated plants before and after the ^{15}N labeling of their nurse plants in control and fungicide-treated patches.

effect) (Table 3 and Fig. 1), thus implying a greater N transfer from the nurse plant to the facilitated plants in patches where the CMNs were intact.

In order to estimate the percentage of ^{15}N transfer, we considered the seven control patches in which the nurse plants showed an enrichment >10‰. The mean percentage of the ^{15}N tracer transferred from the nurse to the facilitated plants was 2.64 ± 1.49% NDFT. Fifteen days after the application of the ^{15}N tracer, some facilitated plants showed foliar $\delta^{15}N$ (Table 2) values which are unlikely to be due to natural abundance fluctuations of the nitrogen isotopic composition of the plants. This provides unequivocal evidence of actual transfer of ^{15}N between the nurse and the facilitated species. That was the case for one individual of the perennial grass *Cathestecum brevifolium* that received 18.9% NDFT. Even without considering this exceptionally high value, the mean percentage of the ^{15}N transferred from the nurse to the facilitated plants was still 1.38± 0.88% NDFT.

4. Discussion

Elucidating the mechanisms that contribute to the persistence of plant facilitative associations in the long term can improve our understanding of plant co-existence and maintenance of biodiversity. Plant facilitative associations will be prone to last if there is a persistent benefit between the adult plants involved. We show that nitrogen can be transferred from the nurse to adult facilitated plants through CMNs, suggesting a mechanism that can contribute to the long-term persistence of plant facilitative associations.

According to our expectations, N transfer is reduced in patches treated with fungicide, which demonstrates that N transfer is to some extent mediated by CMNs. However, our experiment does not tease apart the preferential pathway of N transfer. Several previously proposed mechanisms can result in the observed pattern. For example, (a) nitrogen can be transported from one plant to another directly through hyphal links connecting the roots of both plants (Bethlenfalvay et al., 1991; Frey and Schuepp, 1992, 1993; Johansen and Jensen, 1996), or (b) nitrogen can be released to the rhizosphere by the nurse plant as root exudates, and then be taken up by the fungal hyphae harbored in the facilitated plants roots (Marschner and Dell, 1994; Smith and Read, 2008). Independently of the specific preferential pathway, our experiment shows that in the short term, the N transfer pathway mediated by mycorrhizal fungi is more effective than the "nurse root-soil-facilitated root" pathway (via root exudates by the nurse plant, without fungal mediation).

The detected amount of ^{15}N transfer from the nurse to the facilitated species must be considered as a conservative estimate, especially taking into account the short time allowed for the transfer to occur. Although we find a significant N transfer in just 15 days, several studies show that N transfer from the donor can be accumulated over longer periods, and can amount to up to 40% of total N in the facilitated plant over several months (Høgh-Jensen and Schjoerring, 1997; He et al., 2006; Rasmussen et al., 2007). In addition, the fact that multiple pulses of tracer application increase the amount of N transfer supports its cumulative nature (Gylfadóttir et al., 2007), potentially resulting in large amounts of N transferred in the long-term.

Nevertheless, although comparing ^{15}N transfer estimates requires caution due to different methodologies, scales and inconsistencies in terminology, our results match most of the published estimates, generally showing low transfer values (<10% and often <1%), with just a few exceptions showing higher values (up to 50%) (He et al., 2009; Chalk et al., 2014). Even in controlled microcosm experiments, where donor and receptor plants grow in compartments separated by root-excluding mesh that allows mycorrhizal hyphal passage, the amounts of ^{15}N transfer reported are similar to those observed in this study (less than 4% (Frey and Schuepp, 1992); 0.7–2.5% (Jalonen et al., 2009)). It is not clear how these relatively small amounts of N transfer can influence adult plants fitness. However, it is reasonable to presume that interplant N transfer can be a relevant resource for facilitated plants, especially in semi-arid environments where soil N is a limiting factor and N-fixing legumes are key nurse species structuring plant communities through facilitation (Flores and Jurado, 2003; Bashan et al., 2009; Muro-Pérez et al., 2012). Remarkably, legumes in agro-ecosystems may contribute up to 270–550 kg N ha^{-1} year^{-1} (Sanginga et al., 1994; Jayasundara et al., 1997; Dulormne et al., 2003), mainly due to symbiotic N_2 fixation, which can account for 30–90% of their total N (Giller, 2001). Several field studies have shown that non-legume crops cultivated with legumes may obtain a substantial proportion of their N from the latter (Høgh-Jensen and Schjoerring, 2000; Snoeck et al., 2000; Sierra and Nygren, 2006; Daudin and Sierra, 2008), although little is known about the magnitude of this process in natural ecosystems.

Over long periods of time, the accumulation of small amounts of nutrient transfer can have ecological consequences for both the nurse and facilitated species involved in persistent facilitative interactions. Previous experiments show that the performance of adult plants of the nurse species *M. luisana* (seed production) is not affected by a two-year fungicide treatment when growing alone, but decreased when growing associated to their facilitated plants (Sortibrán et al., unpublished results). This indicates that the reduction in the performance of *M. luisana* is not mediated by the fungicide effects on its own fungal associates, but instead by the fungicide effects on the CMNs connecting *M. luisana* to its facilitated plants. Our results show that CMNs can mediate N transfer between adult facilitated plants, suggesting that nutrient transfer through CMNs might be a potential mechanism allowing persistent benefits for adult facilitated plants.

It is intriguing which evolutionary processes could explain the N transfer from plant to plant or even from mycorrhizal fungi to the facilitated plant, considering that mycorrhizal fungi have a much higher requirement for N than plants (optimal C:N ratio for plant leaf tissue 33:1, for fungal hyphae 10:1; Allen et al., 2003). It has been suggested that the plant-mycorrhizal symbiosis is based on a system of reciprocal rewards that provides both partners with a certain degree of control over the symbiosis by investing more resources on partners that provide more benefits (Kiers et al., 2011). Under this scenario, mycorrhizal fungi might benefit from redistributing nitrogen among their plant partners along source-sink gradients (e.g. from legumes to non-legumes) to ensure the maintenance of multiple sources of carbon, while plants connected by CMNs could mutually benefit from exchanging their less limiting (or surplus) nutrients along source-sink gradients. Nevertheless, much controversy remains regarding the mechanisms that actually govern resource exchange in the plant-mycorrhizal symbiosis (Walder and van der Heijden, 2015).

5. Conclusions

We show that N transfer between adult plants is promoted by mycorrhizal networks. It is known that adult-nurse plants benefit from growing with adult-facilitated plants in our study system (Sortibrán et al., 2014). However, a benefit for the adult-facilitated plants may also favor the long-term persistence of the facilitative interaction. Our results suggest that inter-plant N transfer mediated by CMNs can be a mechanism by which adult facilitated plants continue receiving benefits from their nurse. Further research on the fitness consequences of nutrient transfer between adult plants will be necessary to improve our understanding of the evolutionary implications of facilitative interactions in structuring plant communities and maintaining plant diversity.

Acknowledgments

We thank J.P. Castillo and A. Cerón for help with field samplings. We also thank P. Villar and M. Goberna for revising and providing valuable comments that help to improve the clarity of the manuscript. AMN was supported by a postdoctoral contract from the Ministerio de Economía y Competitividad (FPDI-2013-16266) and an Early Career Project Grant from the BES (3975-4849). Funding was also provided by PAPIIT-DGAPA-UNAM (IN-213414-3).

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Conclusiones generales

El papel de las interacciones bióticas como motores del ensamblaje de la comunidad son cruciales para determinar la membresía de especies (Callaway 2007), siendo la facilitación en gran medida la responsable de la diversidad de especies y su mantenimiento en las comunidades estudiadas (Valiente-Banuet et al. 2006; Valiente-Banuet y Verdú 2007, 2008). El resultado espacial de estas interacciones son la formación de parches (Prentice y Werger 1985; Eccles et al 1999; Castillo et al. 2010), los cuales se forman a partir del establecimiento de una planta nodriza en espacio abierto, con lo cual se da inicio a la entrada de especies y sus interacciones debajo de su copa a través de la interacción de facilitación (Callaway 2007; Broinsten 2009; Castillo y Valiente-Banuet 2010; Castillo et al. 2010). Para el Valle de Zapotitlán y específicamente en la comunidad de Tetechera de *Neobuxbaumia tetetzo*, Mimosa *luisana* es la principal especie nodriza que facilita el 90% de las especies (Valiente-Banuet and Verdú 2008; Cerón 2015).

Un resultado central de este estudio es que *Mimosa luisana,* no sólo facilita el establecimiento de un número importante de especies, sino que al hacerlo también se beneficia al crecer asociada a ellas (Capítulo I, Sortibrán et al. 2014), lo que explicaría la diferencia tan grande de individuos de *M. luisana* creciendo asociadas que creciendo solos (5:1). Nuestros resultados demuestran que *M. luisana* aumenta su adecuación significativamente tanto en cantidad como en calidad de sus semillas cuando crece asociada que cuando crece sola. Más allá de que las interacciones ocurran entre pares de especies, encontramos un efecto positivo en la adecuación de *M. luisana* cuando el parche de vegetación (vecindario filogenético) es más diverso.

Contrario a la idea de que la facilitación cambia a competencia con el tiempo (Tielborger y Kadmon; 2000; Miriti 2006), en este trabajo se muestra por primera vez que la facilitación cambia a mutualismo. Cabe señalar que el mutualismo ya había sido sugerido por otros autores como Eccles et al. (1999) y por Pugnaire et al. (1996). En este último estos autores analizaron el caso de la nodriza *Retama sphaerocarpa* la cual se beneficia de la mejora del suelo producida por sus plantas facilitadas, dando como resultado un incremento de biomasa y del contenido de nitrógeno total, así como potenciales hídricos más altos. Sin embargo, este estudio no abordó el componente reproductivo de la adecuación. A diferencia de lo encontrado por Pugnaire et al. (1996), nosotros no encontramos una relación directa entre el número de especies asociadas y la fertilidad del suelo ni con la disponibilidad de agua. En cambio, sí mostramos que el beneficio mutuo entre plantas vecinas ocurre a través del establecimiento de una red común de micorrizas que permite la transferencia de recursos (Selosse et al. 2006; Simard et al. 2012; Sortibrán et al. 2016; Montesinos-Navarro et al. 2016; Capítulo II).

Estudios posteriores deberán dilucidar los mecanismos específicos que permiten la conexión entre plantas facilitadas por *Mimosa luisana* y el cambio al mutualismo. Nosotros hemos propuesto que *M. luisana*, al ser un nodo hiperconectado a un gran número de micorrizas (Montesinos-Navarro et al. 2012), bien podría en un inicio costear el mantenimiento energético de los hongos micorrícicos. Sin embargo, durante el desarrollo ontogenético de la interacción, las especies facilitadas podrían empezar a abastecer de recursos a la red micorrícica contribuyendo a su mantenimiento, aunado al aumento de la diversidad de hongos que interactúan con un subconjunto de especies facilitadas. Esto es consistente con el hecho de que un rompimiento de las redes de hongos usando fungicidas reduzca la producción del número de semillas por vaina, así como la masa y la viabilidad de las mismas para individuos que crecen asociados a las plantas facilitadas (capítulo II). Esto muestra que el número y la calidad de las semillas son indicadores de cómo la planta asigna recursos según la disponibilidad de nutrientes (Sinclair y Vadez 2002; Marschner 2012; Hulshof et al. 2012; DiManno y Ostertag 2016). Dos líneas de evidencia apoyan la existencia de beneficios mutuos entre *M. luisana* y sus plantas facilitadas a través de un intercambio de nutrientes y agua. Primero, usando isótopos estables ^{15}N, mostrando que los hongos promueven la transferencia de nitrógeno de *M. luisana* a sus especies facilitadas (capítulo III).

Particularmente, para el caso del nitrógeno, los gradientes de nitrógeno natural (N), pueden resultar de la asociación de especies leguminosas (ver Apéndice II figura S2) y no leguminosas, ya que las primeras están en simbiosis con bacterias fijadoras de N_2 y tienen acceso al N atmosférico, inaccesible para otras plantas (Dilworth et al. 2008). Quizás por este hecho, nodrizas como *M. luisana*, juegan un papel importante en la estructuración de comunidades vegetales cuya transferencia de N entre plantas puede ser un recurso importante para las plantas facilitadas, especialmente en los ambientes como el estudiado donde el N del suelo es un factor limitante (Flores-Jurado 2003; Bashan et al. 2009; Sortibrán et al. 2014; Montesinos-Navarro et al. 2016). Incluso *M. luisana* puede transferir más N a especies facilitadas más alejadas filogenéticamente (Montesinos-Navarro et al. 2017). De hecho, se ha sugerido que la simbiosis planta-micorriza se basa en un sistema de recompensas recíprocas que proporciona a ambos socios un cierto grado de control sobre la simbiosis, invirtiendo más recursos en socios que proporcionan más beneficios (Kiers et al., 2011). Las micorrizas arbusculares descomponen y movilizan el N (Hodge et al. 2001). Por lo que los hongos micorrícicos podrían beneficiarse de la redistribución de nitrógeno entre sus socios vegetales a lo largo de un gradiente fuente-sumidero (por ejemplo, de leguminosas a no leguminosas) para asegurar el mantenimiento de múltiples fuentes de carbono, mientras que las plantas conectadas por redes micorrícicas podrían beneficiarse mutuamente de intercambiar sus fuentes menos limitantes (o los excedentes) a lo largo de los gradientes de sumideros.

En conclusión, esta tesis presenta evidencia primeramente que los mecanismos de coexistencia en las comunidades estudiadas son principalmente a través de las interacciones positivas tanto por arriba (facilitación y mutualismo) como por debajo del suelo (micorrizas y bacterias). Así mismo, demuestra por primera vez que las interacciones de facilitación cambian a mutualismo con respecto al tiempo para especies filogenéticamente distantes, una hipótesis que había sido sugerida previamente pero no probada. Además, se presenta evidencia hasta ahora inédita sobre el mutualismo entre plantas, que al ser mediado por terceros interactuantes constituye un aspecto no descrito en la literatura para explicar la coexistencia de especies. Esta información y la obtenida en el último capítulo enfatizan que el microbioma de las plantas juega un papel central en la coexistencia y en el desempeño de los interactuantes. Estos resultados contrastan claramente con aquellos en los que la coexistencia de especies y finalmente la diversidad de una comunidad dependen de la segregación de nichos entre especies. De manera contraria, todo lo obtenido en este estudio muestra que todas las especies interactúan con conjuntos amplios y variados de especies y que las interacciones positivas constituyen un eje conceptual necesario para lograr un entendimiento de los procesos que mantienen la biodiversidad en los ecosistemas.

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