ECOLOGY AND ECO-PHYSIOLOGY OF MYCORRHIZAL SYMBIOSES

IN ARID RIPARIAN AREAS

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A Dissertation

Submitted in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

in Biology

Northern Arizona University

May 2022

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ABSTRACT

ECOLOGY AND ECO-PHYSIOLOGY OF MYCORRHIZAL SYMBIOSES

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LISA MIRA MARKOVCHICK

My dissertation consists of three related research topics focused on translating previous research on mycorrhizal fungi into best practices for natural regeneration, replanting and habitat restoration. In the first chapter, I highlight the ecosystem services known to be provided by mycorrhizal fungi (symbiotic with plant roots), and examine whether utilization of diverse mixes of native mycorrhizal fungi, a potentially crucial tool in land managers' toolboxes to alleviate restoration challenges, has made the translation from academia to restoration practice. In the second chapter, I examine crucial questions surrounding the need for restoring diverse mixes of native mycorrhizal fungi alongside plant communities, and the implications of research on the co-adaptation of mycorrhizal fungi with their symbiont plant hosts for assisted migration. In the final chapter, I investigate whether mycorrhizal restoration and assisted migration can be conducted successfully in tandem with mycorrhizal restoration under by conducting a common garden experiment with reciprocal plantings of local and migrant plant provenances and a migrant mycorrhizal inoculum treatment under harsh, post-*Tamarix* spp. (tamarisk) invasion and drought conditions.

In combination, these three chapters have built on previous research and advanced knowledge of the following. 1) Although the separate, intentional step of mycorrhizal restoration has been shown to have clear benefits if conducted appropriately, and mycorrhizae clearly

support many ecosystem services that are often part of the goals of land management plans, they are still overwhelmingly left out of management planning. 2) Even under ideal circumstances with adjacent natural sources, natural regeneration two-years post planting fails to achieve ectomycorrhizal colonization rates similar to that in natural tree source populations after agricultural disturbance. 3) Natural regeneration of local mycorrhizal colonization is poorer in assisted migrant plant provenances, and concurrent with trends in other ecotype differences such as survival and growth. 4) Under multiple stressors in the field, assisted migrant plant provenances given an appropriate mycorrhizal inoculum can survive at higher rates than local plant provenances. 5) Inoculation with a contrasting ecotype's live mycorrhizae can have neutral to negative effects that interact with site conditions. 6) The afterlife of an invasive species in the soil once the invasive plants have been removed can have dire consequences for native plants after restoration. Below is a summary of each of the three chapters.

Chapter 1: During the coming decade, planting material shortages are expected to constrain restoration. Climate change will reduce recruitment and exacerbate the need for restoration and natural regeneration. Concurrently, research shows that native mycorrhizal fungi optimized to plant provenance and site conditions significantly and meaningfully accelerate restoration, support crucial ecosystem services, and provide natural climate solutions (sequestering carbon), and nature-based solutions for climate change (providing climate adaptation). We reviewed 130 systematically available management plans for natural areas in the United States to evaluate whether the science-based innovation of restoring native mycorrhizal communities has translated into implementation. Results are notable: management plans frequently discussed the ecosystem services mycorrhizal fungi provide, but nearly one half (46%) viewed fungi solely as pathogens or ignored them altogether. Only 8% of plans mentioned

mycorrhizae. Only one plan mentioned that mycorrhizae were potentially helpful to natural regeneration, and one mentioned utilizing soil as a restoration tool. Our examination of publicly available data and case studies suggests that relatively meager protections and research funding, research difficulty and data paucity, and limited access to mycology experts and training characterize this gap between science and implementation. A database of literature showcasing mycorrhizal ecosystem services and benefits is provided to highlight when and why mycorrhizae should be considered in management, regeneration, and restoration. Three action items are recommended to safeguard native mycorrhizal communities and accelerate restoration and regeneration. Ten implementation tips based on scientific literature are provided to clarify the need and methods for mycorrhizal restoration.

Chapter 2: Many factors deplete and shift the mycorrhizal fungal communities upon which native plants depend, and consequently their often sizable and meaningful benefits to plant survival and growth, and a variety of ecosystem services. Yet, as the UN Decade on Ecosystem Restoration begins, active restoration of native mycorrhizal fungal communities is often overlooked as a strategy to enhance and sustain ecosystem restoration. Few studies focus specifically on implementation questions, such as the success and timeliness of natural mycorrhizal regeneration in disturbed soils and its effect on replanting success. Simultaneously, assisted migration of plant species or provenances has been suggested to hasten ecosystem climate change adaptations, but the consequences of assisted migration for mycorrhizal regeneration and associations remain poorly described. To determine the success of natural mycorrhizal regeneration, its interaction with plant provenance, and the consequences for restoration success, we evaluated naturally regenerating mycorrhizal colonization of *Populus fremontii* (Fremont cottonwood), a foundation riparian tree, in trees from one local and two

assisted migrant source populations two years after planting. Three main lessons emerged. 1) Ectomycorrhizal colonization rates two years post-planting were considerably lower than those found in source populations, even under ideal conditions with an adjacent source of mycorrhizal host trees. 2) Within the planting site, proportions of dedicated exchange sites between plants and mycorrhizal fungi closely followed plant provenance, with trees from greater distances demonstrating fewer EMF root tips and AMF arbuscules. 3) For the plant provenance struggling the most, a close association between tree survival rates and mycorrhizal colonization was found. Results suggest that active mycorrhizal restoration and optimizations of pairings between fungal, plant, and site provenances could maximize investments in restoration and natural area management, and help offset challenges posed by shortages in planting material and climate change.

Chapter 3: Under climate change, the need for and importance of natural regeneration, replanting, and restoration is expected to grow while planting and recruitment success decrease, and planting material shortages continue to constrain replanting efforts. Thus, strategies to optimize restoration effectiveness seem increasingly important. Assisted migration and the restoration of native mycorrhizal communities appear promising. However, intraspecies assisted migrations often still show reduced survival compared to local provenances, and mycorrhizal inoculation, effective if well-matched to plants and site conditions, can have neutral to negative results with poor pairings. Few studies examine the interaction between these two strategies and invasive species soil legacies, particularly under drought conditions exacerbated by a warming, drying climate. To evaluate these interactions, we planted *Populus fremontii* (Fremont cottonwoods) from two ecoregions (local and warmer) in soils with and without legacies of invasion by *Tamarix* spp. (tamarisk), and with and without restoration of native mycorrhizal

fungi. Four main lessons emerged. 1) The soil legacies left behind after tamarisk invasion reduced survival by 85%. 2) Actively restoring a diverse, native community of mycorrhizal fungi after tamarisk invasion doubled and then tripled survival during the first and second field seasons, respectively. 3) Inoculation sometimes had neutral and even negative effects, interacting with timing, pairing between tree and inoculum sources, and site conditions. 4) During the second field season, assisted migrants survived at twice the rate of trees from the local ecoregion, if inoculated with an appropriate and diverse native mix of mycorrhizal fungi. Results emphasize the detrimental effects of soil legacies left behind after tamarisk invasion, the beneficial effects of appropriate mycorrhizal restoration, and the need to thoughtfully optimize pairings between plants, fungi, and site conditions.

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DEDICATION

As I write this, aggression and chaos have again erupted in the eastern European region bordering Russia from which all of my ancestors fled in the late 1800s. Responding to Putin's aggression, the bravery and kindness of the people of Ukraine and nearby nations has set the hearts of the world's people ablaze. As we see their heroism, we are learning what true courage looks like.

This work is dedicated to Ukraine, to my husband and his family for looking after me and teaching me what it is like to be part of a loving, functional family, and to my own family divided by mental illness, who try their best.

PREFACE

Chapters in this dissertation have been and are being submitted for publication in the peer-reviewed journal, Restoration Ecology. Therefore, the formatting of each is consistent with Restoration Ecology's author guidelines. Each chapter is/will be submitted with the co-authors stipulated in each chapter. Thus, I often use the term "we" instead of "I".

Chapter 1: The gap between mycorrhizal science and application: existence, origins, and relevance during the United Nation's Decade on Ecosystem Restoration

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Abstract

During the United Nation's Decade on Ecosystem Restoration, planting material shortages are expected to constrain restoration. Climate change will reduce recruitment and exacerbate the need for restoration and natural regeneration. Concurrently, research shows that native mycorrhizal fungi optimized to plant provenance and site conditions significantly and meaningfully accelerate restoration, support crucial ecosystem services, and provide natural climate solutions (sequestering carbon), and nature-based solutions for climate change (providing climate adaptation). We reviewed 130 systematically available management plans for natural areas in the United States to evaluate whether the science-based innovation of restoring native mycorrhizal communities has translated into implementation. Results are notable: management plans frequently discussed the ecosystem services mycorrhizal fungi provide, but nearly one half (46%) viewed fungi solely as pathogens or ignored them altogether. Only 8% of plans mentioned mycorrhizae. Only one plan mentioned that mycorrhizae were potentially helpful to natural regeneration, and one mentioned utilizing soil as a restoration tool. Our examination of publicly available data and case studies suggests that relatively meager protections and research funding, research difficulty and data paucity, and limited access to mycology experts and training characterize this gap between science and implementation. A database of literature showcasing mycorrhizal ecosystem services and benefits is provided to highlight when and why mycorrhizae should be considered in management, regeneration, and restoration. Three action items are recommended to safeguard native mycorrhizal communities and accelerate restoration and regeneration. Ten implementation tips based in scientific literature are provided to clarify the need and methods for mycorrhizal restoration.

Implications

- Native mycorrhizal fungi (symbiotic with plant roots) customized to plant provenance and site conditions meaningfully boost restoration outcomes, ecosystem services, natural climate solutions, and nature-based solutions for climate change.
- Management plans overwhelmingly discuss the services to which mycorrhizae contribute, but fail to mention using mycorrhizal fungi for regeneration or restoration.
- Closing this gap between mycorrhizal science and application could accelerate restoration and regeneration, and help mitigate climate change and planting material challenges.
- A database of mycorrhizal benefits is provided to assist identification of when mycorrhizae should be considered in management and restoration.
- Action items and implementation tips for natural areas are provided to assist restoration practitioners and managers of natural areas.

Introduction

As the United Nations' (UN's) Decade on Ecosystem Restoration begins, studies suggest restoration will be limited by supplies of plant propagative material (National Academies of Science, Engineering, and Medicine 2020; Fargione et al. 2021). Areas in need of restoration and regeneration are expected to grow due to ongoing deforestation, shifting agriculture, and the increasing frequency and severity of extreme weather events (e.g. Curtis et al. 2018; National Academies of Science, Engineering, and Medicine 2020; Fargione et al. 2021). Simultaneously, natural regeneration (Chazdon 2017) and recruitment are being complicated and reduced by the impacts of climate change (Panetta et al. 2018; Costa dos Santos et al. 2019; Garnier et al. 2021). Offering a potentially crucial offset to these challenges, studies and meta-analyses examining the restoration of mycorrhizal fungi demonstrate the efficacy of this innovation when it is appropriately applied (e.g. Rua et al. 2016; Koziol & Bever 2017; Neuenkamp et al. 2019).

For example, restoring native mycorrhizal communities in conjunction with native plant communities can increase planting survival and establishment, biomass, species richness, and similarity to reference ecosystems. Improvements of 30% or more are not unusual when diverse mixes of native mycorrhizal fungi custom-paired with the plants and site conditions are utilized (e.g. Koziol & Bever 2017; Neuenkamp et al. 2019).

The need for and effectiveness of mycorrhizal restoration appears to arise from the combination of two factors. First, disturbances including land use changes, invasive vegetation, pollution, reduced populations of native plant hosts, and herbicide application can negatively impact native mycorrhizal communities in ways that can last for years (e.g. Meinhardt & Gehring 2012; Koziol & Bever 2017; Helander et al. 2018). Second, mycorrhizal communities that occur on plant propagative material do not seem sufficiently

optimized to plant host and planting site conditions, even when specific attention is given to this step (e.g. Southworth et al. 2009, Sykorova et al. 2007, Moreira et al. 2007). For example, oak seedlings growing at a natural site and seedlings from the same site grown in a greenhouse shared none of the same mycorrhizae (Southworth et al. 2009). For these reasons, restoring mycorrhizal fungi optimized for planting site and plant provenance as a separate step could help offset other restoration, reforestation and land management challenges.

The management and restoration of mycorrhizal fungi is also appropriate due to their own merits, since mycorrhizal fungi provide a variety of ecosystem services that increase the function, resiliency, and positive impact of natural areas (Costanza et al. 1997). The success and results of mycorrhizal symbioses are species, provenance, and context dependent (e.g. Rillig & Mummey 2006). Additionally, only a small subset of any mycorrhizal fungal community may provide a specific service (e.g. Egerton-Warburton et al. 2007). Thus, it might be expected that a relatively small proportion of studies would find any one specific benefit. Yet, the scientific literature reveals many meaningful contributions of mycorrhizal fungi to ecosystem services. Figure 1 visually summarizes some of the ecosystem services mycorrhizal fungi are known to provide (left), and the corresponding results of their depletion (right). We summarize these ecosystem services here (see key examples and references in Supplement S1), before investigating whether a gap between the science and implementation exists.

Mycorrhizae improve plant nutrition, build fertile soil, and improve moisture infiltration and retention. Plant reproduction, including flower number and mass, nectar production, and seed production, can be improved by mycorrhizae. Mycorrhizal hyphae and secretions cycle nutrients and aggregate soil. They protect against erosion, reduce nutrient loss and leaching (see examples and references in Supplement S1).

Contributions of mycorrhizae to ecosystem services also seem particularly relevant under climate change. Mycorrhizae serve as natural climate solutions (increase carbon storage and/or avoid emissions; Griscom et al. 2017), sequestering carbon underground in hyphae, competing with decomposers to reduce carbon release, and improving above ground carbon storage through increased plant growth. Mycorrhizae also act as nature-based solutions for climate change (natural means of providing climate mitigation and adaptation; Nesshöver et al. 2017). By mediating plant water use and access, mycorrhizae improve plant drought resiliency and contribute to shade and canopy transpiration and cooling (for example, Wu & Xia 2005). Mycorrhizae increase flowering duration, reducing potential timing mismatches between plants and pollinators under climate change (Lu & Koide, 1994; Botham et al., 2009; Barber & Gordon, 2015). While invasive vegetation and pests are expected to increase, mycorrhizal fungi boost plant defenses to pests and pathogens (Gregory et al. 2009; Clements and Ditommaso 2010). While the nutritional value of crops is expected to decrease in response to climate change, mycorrhizae increase their nutritional value (Avio et al. 2018; Bisbis et al. 2018). By distributing resources and warning signals through underground networks, mycorrhizae spread resources and risk, improving ecosystem stability and resiliency similar to this function in investment portfolios (Schindler et al. 2015). Mycorrhizal fungal diversity also supports other biodiversity and productivity in non-additive ways. For example, the efficiency of resource use increases with increasing mycorrhizal fungal diversity (van der Heijden et al.1998).

Although more rarely studied, these mycorrhizal contributions cascade through ecosystems, influencing habitat quality and resource availability for other species in non-additive ways. For example, one study estimates that biomass in the Serengeti (including large carnivores) would be halved without the phosphorus supplied by mycorrhizal fungi

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(Stevens et al. 2018). Another study suggests that adding appropriate mycorrhizal inoculation to restoration for a 6.89 hectare site would increase endangered bird habitat from 0 to 1.2 hectares six years post-restoration (Tracy & Markovchick 2020).

Others have noted there is frequently a gap between science and implementation in conservation (Game et al. 2015). Thus, we questioned whether the utilization of mycorrhizae optimized for plant provenance and site conditions as an innovation in restoration had successfully bridged this gap. We hypothesized that usage of this tool in restoration and land management may still be relatively uncommon outside academia. To investigate this issue, we examined natural resource management plans in the United States (US) available online from two different sources for their treatment of ecosystem services and mycorrhizae.

Review of management plans

We downloaded and systematically searched 130 national forest and national grassland management plans, and state forest action plans. We chose these plans because they cover many acres of natural resources throughout the US (over 853 million for state forest action plans, and over 97 million for national forest and grassland management plans), represent habitats populated by two of the most common mycorrhizal fungi (arbuscular mycorrhizal fungi and ectomycorrhizal fungi), and are systematically available, avoiding bias due to inconsistent availability (National Association of State Foresters 2019; United States Forest Service 2019). Management plans from the National Association of State Foresters (n=55) were typically more recent, produced from 2008 to 2018 (with a median year of 2010), while plans downloaded from the United States Forest Service (n=75) were often older, produced between 1984 and 2019 (with a median year of 2004). Additional methodological details and the resulting database can be found in Supplement S2.

To evaluate each management plan's concern with the life-sustaining ecosystem

service categories outlined by Costanza et al. (1997), we searched each management plan (n=130) for terms related to each ecosystem service category (Table 1) and reviewed each occurrence to confirm the intent of the word usage. To evaluate the consideration of mycorrhizal fungi in each management plan, we systematically searched each management plan (n=130) for terms related to mycorrhizae (Table 2). Since very few plans mentioned mycorrhizae, we expanded the search to include terms related to multiple kinds of fungi (as in Table 2). Each occurrence was read to confirm its intent and categorize each mention with regard to its view of fungi (as described in Table 2 and Supplement 2).

Treatment of ecosystem services and fungi in management plans

Management plans were largely concerned with the same ecosystem services to which mycorrhizae contribute: 85-99% of the plans discussed ecosystem services in eight out of ten categories. For the remaining two categories of ecosystem services, 72% of plans discussed climate issues and 27% discussed pollination (n=130, Figure 2; Supplement S2). Similar to a study in Australia that found 30% of national park management plans made no mention of fungi (Pouliot 2013; Irga et al. 2018), we found 22% of plans made no reference to fungi. Twenty-five percent (25%) viewed fungi solely as a threat. This percentage is substantially lower than the 90% of plans that viewed fungi solely as a threat found by Pouliot in 2013, but still means that out of 130 management plans, nearly half (46%; 60 of 130) either completely ignored the entire Kingdom Fungi or viewed fungi only as a threat without any reference to their utility, contributions or conservation. As Figure 2 shows, when management plans mentioned a fungal term (n=102), it was most frequently due to disease (82%), mushrooms as food and harvestable items (56%), or lichens as air quality indicators and wildlife food (54%). Even plans that mentioned fungi only rarely mentioned mycorrhizal fungi (11%) or fungal endophytes (non-pathogenic/symbiotic fungi found inside plant

tissues; 2%).

Nearly all plans (92%; and even 89% of those mentioning fungi) failed to mention mycorrhizae (Figure 2). Only 8% of plans made a reference to mycorrhizae (n=11). Only one plan specifically mentioned that mycorrhizae were potentially helpful to natural forest regeneration, and only one plan mentioned utilizing soil as a restoration or regeneration tool. Most plans that mentioned mycorrhizae (91%) were from the United States Forest Service (with the exception of the Wisconsin Statewide Forest Strategy). We hypothesized that more recent plans might be more likely to mention mycorrhizae since the most convincing meta-analyses on the benefits of mycorrhizae to restoration and land management tend to be more recent. However, plans that mentioned mycorrhizae were developed between 1989 and 2015, with a median year of 2005 (similar to the median of 2004 seen for United States Forest Service plans overall). With the exception of the Wisconsin Statewide Forest Strategy, plans mentioning mycorrhizae were from the western United States (e.g. WA, CA, OR, ID, MT, WY), and 36% fell under the Northwest Forest Plan Survey and Manage Guidelines which required the inclusion of fungi in monitoring and management (Molina 2008; Davoodian 2015).

Potential causes of the science-application gap

Few studies have evaluated disconnects between science and application for beneficial fungi, but those studies reveal that fungi are often ignored except as pathogens in public perception, university courses, and even by land managers and biologists (Pouliot 2013; Irga et al. 2018). To better understand the nature of this gap, we investigated factors we hypothesized could inhibit the translation of science on beneficial fungi into application, relative levels of: regulatory protection and research funding, research difficulty and cost, and access to fungal expertise and training in restoration and land management contexts.

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Relative protections and research funding

Detailed knowledge of, and experts focused on, specific taxa in the restoration practitioner and natural resource management workforce are frequently driven by legal protections such as the Endangered Species Act (ESA) and research funding. Thus, we hypothesized poor protections and research funding might contribute to implementation challenges for mycorrhizae. To better understand the role regulatory protections might play, we systematically searched publicly available data on protected species for fungal taxa (see Supplement S3 for detailed methods; Figure 3). Fungi are an estimated 7.4% of the biodiversity on Earth and an estimated 97-99% remain to be discovered (Larsen et al., 2017; Figure 3). Yet, our systematic search of the World Conservation Union's Red List (World Conservation Union 2019; IUCN) and US Endangered Species Act list (United States Fish and Wildlife Service 2019a; ESA) revealed that fungi are rarely mentioned in conservation listings (0-0.09%; Figure 3; Supplement S3; Table S1). Indeed, analyses of the ESA suggest that coverage of organisms other than plants and animals (which are explicitly covered) ranges from inconclusive to unlikely (Davoodian 2015). European monitoring efforts for fungi with visible mushrooms (macrofungi) show similarly sparse protections, despite recent commitments to, and progress in, reviewing the conservation status of additional fungal taxa under the World Conservation Union's Red List (Senn-Irlet et al. 2007; May et al. 2018; Supplement S3).

To investigate whether fungal research funding levels might also be contributing, we systematically searched fiscal year (FY) 2018 federal research projects for mentions of different terms related to fungi (see Supplement S3, Tables S2 and S3, for details). Although approximately 5% of FY2018 federal research projects and dollars that mention organismal groups mention fungi, approximately 70% of this fungal research funding appears to relate to

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disease (Figure 3). Fungal pathogens are serious threats, and their impact is expected to increase with climate change (Almeida et al. 2019). Yet, beneficial fungi are likely more numerous than fungal pathogens. For example, pathogenic fungi appear to constitute a small proportion of the fungal soil communities in North America compared to beneficial ectomycorrhizal and decomposer fungi (59 compared to 1988 taxa; Tedersoo et al. 2014). These beneficial fungi could be our allies against disease, pests, and stressors. Many fungi produce antibacterial and antifungal compounds (Xu et al. 2015). Even fungi initially viewed as solely pathogenic are sometimes found to have beneficial qualities, such as producing key anticancer compounds (Stone et al. 1993). Yet, only 1.2% of US FY2018 organismal research dollars for projects that mention fungi refer to mycorrhizal fungi, for example.

Relative research difficulty and cost

Given the complexity of fungal biology (tissues intermingled with symbionts, numerous mating types/genders, microscopic to vast underground sizes, etc.) we hypothesized that the difficulty and cost of fungal research might also lead to challenges translating science into management. The study of mycorrhizae frequently requires field collections, short- and long-term storage and curation, microscopy, culturing, genomic sequencing and stable isotope and mass spectral analyses (Lindahl et al. 2013). Even questions of classification can be elusive for these cryptic organisms. Species must often be defined statistically as a percentage of genomic sequence match, due to challenges associated with observing breeding behavior and other factors typically used for more charismatic taxa (Lindahl et al. 2013). Additional complexity occurs because some fungi may not exist independently of their hosts, and fungal functions, persistence, and responses vary with changes in symbiont and environment (e.g. Rillig & Mummey 2006).

To evaluate mycorrhizal research difficulty and the role it could play in preventing

effective translation of scientific promise to conservation and management, we quantified the differences involved in surveying for fungi versus plants using a specific case study from the experience of one of the authors (Figure 4). This example, surveys for an orchid, *Platanthera cooperi,* and its orchid mycorrhizal fungi (OMF), was chosen for three reasons. 1) This example consists of a single project with consistent context and oversight. 2) In this example, both plants and mycorrhizal fungi must be searched for utilizing their respective methods. 3) Clear cost estimates exist for both the plant-centered and mycorrhizal fungi-focused portions of the project. The estimates provided focus on surveying the presences and identities of plant and fungal taxa, excluding other conservation and management actions. A detailed narrative of Figure 4 and explanations of funding estimates can be found in Supplement S4. Results show that despite the crucial importance of OMF to the plant, there is a relative lack of information regarding OMF requirements concurrent with large disparities in survey cost and effort (Figure 4).

Despite the potential appeal of monitoring and managing the plant host alone, science is clearly identifying the insufficiency of this approach in many contexts. For example, restoration, regeneration (and range shifts in response to a changing climate) could depend entirely on the presence and optimization of fungal symbioses, and whether symbionts have been eliminated from the soil by invasive species, pollution deposition, disturbance or herbicide application (Meinhardt & Gehring 2012; Helander et al. 2018). Koziol and Bever (2017) found some late successional plant species could not be restored without concurrent restoration of the appropriate mycorrhizal fungal community. Multiple studies now indicate that appropriate pairings between plants, soil, and mycorrhizal fungi are key for beneficial results (Johnson et al. 1992; 2010; Rua et al. 2016). Both intraspecific and interspecific mycorrhizal diversity is crucial to outcomes. For example, biomass responses to intraspecific

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diversity was similar to that from interspecific diversity in ectomycorrhizal fungi, and both had a stronger effect than fourfold differences in abiotic factors (Wilkinson et al. 2010; Johnson et al. 2012). Similarly, other studies have found that mycorrhizal diversity determines plant biodiversity and ecosystem productivity (e.g. van der Heijden et al. 1998).

Yet, lower levels of regulatory protection and research funding can often lead to reduced levels of data availability for the types of information needed to translate science into application, particularly when coupled with relatively difficult and costly research. For example, information typically required for conservation and management of a taxon includes population sizes, distribution, trends and threats (Table 3). These types of information often require repeated monitoring across time and a taxon's range. Such efforts generally do not exist for fungal taxa, especially less visible taxa such as mycorrhizal fungi.

Fungal research funding levels presented above (Figure 3, Supplement S3, Tables S1-S4) are relatively low. If relative fungal diversity (Figure 3, Table S1) and difficulty and cost of fungal research (Figure 4, Supplement S4) are considered in concert, relative funding for fungal research is even more incongruent (see Supplement S5 and Table S5 for details). Decisions about the prioritization of research dollars requires a separate and thorough consideration of societal values and the effectiveness of specific strategies. However, we offer this example to inform expectations and efforts with regard to translating science into application for beneficial fungi. For example, awaiting more thorough knowledge for implementation of mycorrhizal restoration (without meaningful changes in protections or research funding) could come with significant opportunity costs and loss of mycorrhizal diversity.

Relative access to fungal training and expertise

Clear mandates for species protection and research funding often drive job and training availability, and the incorporation of experts into the practitioner workforce. We examined the presence of mycologists focused on beneficial fungi in the workforce using the handbook of federal position classifications (United States Office of Personnel Management 2018). Although there is overlap between categories, this examination reflects that of federal natural resources job classifications (n=30), 33% relate to animals, 40% relate to plants, and only one category (3%) relates neutrally or positively to microbiology (covering fungi, bacteria, archaea and more). At least two categories (6%) relate specifically to fungi as threats. The few studies we could locate that address this issue clearly state that mycologists and mycological knowledge are a relative rarity among natural resource managers (Molina 2008; Davoodian 2015; Irga et al. 2018). Irga et al. (2018) noted that out of 11 universities in New South Wales, Australia, only three had a course on mycology or fungi. Molina (2008) and Irga et al. (2018) note that mycologists often do not have experience in restoration and management, and restoration and land management practitioners often have little access to mycologists. The exception seems to have been the integration of mycologists and mycological protection into land management under the Survey and Manage Standards and Guidelines of the Northwest Forest Plan (Molina 2008; Davoodian 2015), which required inclusion of fungal monitoring and management. This effort ended with legal cases over the nature of integration between protections and industry. However, this effort resulted in twelve years of monitoring 9.7 million hectares, yielding approximately 14,400 fungal records. This suggests great strides could be made in translating fungal research into land management if government, private, and non-profit organizations designated positions for mycologists specializing in beneficial fungi as they often do for botanists, physical soil scientists, and wildlife biologists (Molina 2008).

Reasons for concern

The gap between mycorrhizal science and implementation would be less concerning if data suggested fungi were flourishing. However, the opposite is true. For example, the Survey and Manage Standards and Guidelines of the Northwest Forest Plan found that 55% of the 234 fungal taxa included in the program were found at fewer than 20 locations, and 42% were found at 10 or fewer sites (Molina 2008). For comparison, the Eastern prairie fringed orchid (*Platanthera leucophaea*) is extant in only 59 populations and listed as threatened under the ESA (United States Fish and Wildlife Service 2019a). Its relative, the chaparral rein orchid (*Platanthera cooperi*) from Figure 4, is found at 162 different locations (The Calflora Database 2022) and is still considered vulnerable but not protected by the Endangered Species Act (ESA). The decline of mycorrhizal fungi, while more difficult to assess than macrofungi due to their largely unseen, cryptic nature, is reported in many studies due to various causes including land use change, invasive species, pollution deposition, and herbicide use (Meinhardt & Gehring 2012; Swaty et al. 2016; Helander et al. 2018).

In some cases, the dangers facing beneficial fungi mirror those for other species and the same conservation strategies used for more charismatic species could be a successful strategy (Minter 2011). For example, Clemmensen et al. (2013) found that habitat fragmentation, a common threat to biodiversity, is also a concern for mycorrhizal fungi and conservation mycology. Thus, conservation programs targeting the mitigation of fragmentation could benefit both charismatic taxa and lesser known taxa like mycorrhizal fungi. However, Cameron et al. (2019) documented geographic mismatches between terrestrial aboveground and soil (including mycorrhizal) biodiversity, finding that these mismatches cover 27% of the earth's terrestrial surface. Thus, efforts to protect areas of aboveground biodiversity may not sufficiently reduce threats to soil biodiversity (Cameron et al. 2019). Additionally, even within areas that are protected and where aboveground and soil

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biodiversity overlap, disturbances such as the treatment of invasive vegetation with pesticide (Helander et al. 2018), or self-reinforcing soil legacies left after invasion by exotic vegetation (e.g. Meinhardt & Gehring 2012), may quietly continue to reduce beneficial fungi, if these impacts are not recognized and addressed (Davoodian 2015; Kew Royal Botanical Gardens 2018; May et al. 2018).

Implications for research

Given the status and funding of fungal knowledge, it seems crucial to consider what kinds of fungal research could best support translation of science on beneficial fungi into implementation. Here we focus on mycorrhizae. We suggest that research identifying the mycorrhizal ecosystem service dollar values, and research explicitly exploring their effects on other ecosystem members, are crucial to informing a more widespread understanding of the costs and benefits of implementation. Costanza et al. (1997) argued that being aware of the dollar values of naturally provided ecosystem services aids in better decision-making. This is just as true for mycorrhizal fungi as for other taxa. Yet, a GoogleScholar™ search of Ecosystem Services journal articles with the term "dollar" yielded 226 results. A similar search using the term "dollar" and mycorrhizal terms yielded three results (Supplement S6 and Figure S1). Only one of these results was an Ecosystem Services journal article mentioning the ecosystem services of mycorrhizae, briefly mentioning mycorrhizal nutrient uptake and erosion reduction with no valuation of these services (Supplement S6). Research regarding the links between mycorrhizae and benefits to protected areas or the success of protected or commercially valuable species could appropriately inform the conservation of multiple species and integrate mycological concerns with those of desired ecosystem services, nature-based solutions, and natural climate solutions. These types of research also lay a foundation for mycorrhizae to be considered in payment for ecosystem service and

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carbon credit programs (Fripp 2014; Senadheera et al. 2019), which could improve the conservation and restoration of mycorrhizal fungi while increasing ecosystem services and carbon sequestration.

Although we have focused on mycorrhizae here, it is clear that other taxa are similarly understudied and underserved in the implementation of conservation and land management. For example, endophytes were mentioned in management plans even less frequently than mycorrhizae despite promising research on their contributions. Our investigation of research funding and regulatory protections revealed that Archaea, Chromista, Protozoa, and Bacteria levels of diversity and research funding appear similarly incongruent. Issues surrounding fungi with regard to regulatory protections and geographic mismatches between aboveground and belowground biodiversity (Cameron et al. 2019) seem similarly applicable to these other Kingdoms. Adequately covering these mismatches would require expertise and space well beyond this article. However, we have tried to provide examples of methods that could be utilized to identify, illuminate, and inform efforts at narrowing gaps between science and application regardless of target taxa.

Conclusions and implementation tools

There is some good news for fungi. World Conservation Union Red List reviews of fungi are increasing (Supplement S3). Kew Botanical Gardens has issued inaugural (and follow-up) State of the World's Fungi reports and piloted citizen scientists' use of mobile sequencing sample preparation technology (e.g. Harries 2018; Kew Royal Botanical Garden 2018). MycoFlora (2020) and MycoPortal (2020) pair citizen scientist specimen submissions with professional genomic sequencing. While these programs do not address challenges associated with identifying fungal functions or more invisible fungal forms, they represent important steps to reduce costs and increase available information (Dickinson et al. 2010;

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Irga et al. 2018).

Still, the difficulty and state of fungal science, relatively low levels of fungal protections and research funding, and the relative rarity of mycological experts among practitioners pose barriers to maximizing fungal contributions to management and conservation for all species. This is true seventeen years after *Science* magazine's special issue focused on soils and fungi as the final frontier (Pennisi 2004).

Given the relative rarity of mycologists (Senn-Irlet et al. 2007; Irga et al. 2018) increasing links between land managers, restoration practitioners, citizen scientists, and mycologists is crucial, since this has improved data coverage for so many other disciplines (Minter 2011; Davoodian et al. 2015). Improving integration among mycology and other disciplines has policy implications as well. For example, efforts to mandate and fund increased restoration and regeneration could include explicit calls for improved integration of appropriate mycorrhizal restoration.

While there is much science still needs to learn about fungi and mycorrhizae, evidence is fairly clear regarding the efficacy of restoring diverse native mycorrhizal communities optimized to plant provenances and site conditions (e.g. Neuenkamp et al. 2019; Rua et al. 2016; Maltz & Treseder 2015). Evidence also seems fairly clear regarding the gap between science and implementation on this topic. To advance implementation, we have developed three tools for restoration practitioners and land managers, included in the supplemental information (Supplements S1 and S7).

To empower practitioners to advocate for the assistance needed for implementation, and enable examination of when mycorrhizae should be considered in land management and restoration, we created a database of studies showcasing mycorrhizal ecosystem service contributions and restoration benefits (Supplement S1). The database lists examples by

ecosystem service category, provides references for each example, and summarizes the treatments compared, type of effect, and magnitude of effect seen in the example.

Based on the scientific literature, we recommend three mycorrhizal action items and 10 mycorrhizal implementation tips for natural areas (in Table 4). The full versions of these tools include references and more detailed logic (Supplement S7). The mycorrhizal action items suggest three ways that restoration practitioners and managers of natural areas can benefit mycorrhizal fungi and the health of their ecosystems by considering mycorrhizae in their restoration and planning efforts. The mycorrhizal implementation tips navigate and summarize some of the points we've discussed throughout this article, but in a condensed format specifically designed with practitioners in mind. We begin the implementation tips by highlighting the benefits that mycorrhizal fungi provide and the important role of their diversity (e.g. Supplement S1; van der Heijden et al. 1998; Johnson et al. 2012), step through the reasons why mycorrhizae need restoration, and summarize the evidence demonstrating what their successful restoration achieves (e.g. Meinhard & Gehring 2012; Helander et al. 2018; Neuenkamp et al. 2019). We conclude the implementation tips with details to consider for appropriate and successful mycorrhizal restoration, including the poor performance and ethical considerations of mass-produced products, neutral to negative results seen with poor pairings (e.g. Maltz & Treseder 2015; Rua et al. 2016; Saloman 2022), and factors to consider to achieve successful inoculation (e.g. Mortimer et al. 2005; Walker 2003; Supplement S7).

As the UN Decade on Ecosystem Restoration begins, appropriate mycorrhizal restoration can help offset restoration challenges, and represents a critical step towards re-orienting ecosystem restoration around whole ecosystems from the ground up.

Acknowledgements

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Tables and figures

Table 1.1: Simplified ecosystem service (ES) categories used here, the original categories identified by Costanza et al. (1997), and search terms for identifying management plan mentions of each category. For manageability we focused on biological and life-sustaining categories (omitting cultural ecosystem services).

ES categories reviewed here	Costanza et al. (1997) categories	Search terms used
Disturbance regulation	Disturbance regulation	adapt, adapting, adaptation, buffer, drilling, extreme weather, fire, flood, mining, natural disaster, natural hazard, plasticity, resilience
Climate	Climate regulation Gas regulation	carbon credit, carbon sink, carbon market, carbon storage, climate change, climate regulation, sea level rise, sequester, carbon
Water	Water regulation Water supply Waste treatment	clean water, filter, water availability, water quality, water quantity, water regulation, water supply, waste treatment
Habitat & biodiversity	Refugia	biodiversity, biological diversity, ecosystem, endangered species, foundation species, protected species, restoration, species diversity, threatened species
Genetic resources	Genetic resources	genetic(s), genetic adaptation, genetically adapted, genetic diversity, genetic exchange, genetic transfer, genetic variation
Nutrient cycling	Nutrient cycling Soil formation	soil formation, soil quality, quality soil, soil productivity, fertile soil, nutrient cycle, nutrient cycling soil recycling, soil conservation, conserve soils, conservation of soil resources, ability of soil to hold nutrients, decomposition
Erosion control	Erosion control/ Sediment retention	air quality, erosion, grazing
Pest regulation	Biological control	bark beetle, disease, exotic, herbivory, invasive, pest
Pollination	Pollination	pollinate, pollinator
Raw materials	Food production Raw materials	agriculture, food, harvest, regeneration, succession, tree age, wood product

Table 1.2: The main questions regarding the treatment of fungi in state and federal management plans systematically available on the internet, and the methods and search terms used to address them.

Questions Addressed	Search Terms / Methods
1) Are ecosystem services to which fungi contribute of concern in management plans? 2) If so, which ecosystem services are of concern?	Table 1 contains the ecosystem service categories considered. Each plan was searched for terms related to each ecosystem service category ("extreme weather" for climate regulation, see Table 1).
3) Are mycorrhizae mentioned in management plans? 4) Are fungi mentioned?	Each plan was searched for terms relating to mycorrhizae (with poor results), and then for any terms relating to fungi, including: fungal, fungi, fungus; mycorrhiza, mycorrhizae, mycorrhizal; mushroom; endophyte; lichen; microbe, microbial, microbiome.
5) If fungi appear in the plan, are they merely discussed, are they surveyed for, or is some action taken or prescribed?	Mentions of fungi were classified as a) discussed, b) surveyed, or c) managed.
6) Do fungi appear in the plan as a threat; a resource, a solution, or indicator; both?	Each mention of a fungal term was classified as a: a) threat, or b) resource, solution, or indicator. Plans that included both kinds of mentions were categorized as both.

Table 1.3: Types of information typically required for conservation and management of taxa.

Source	Information needed for conservation & management
Endangered Species Act (United States Fish and Wildlife Service 2019b)	• current or expected habitat decline for the taxa • overutilization data • disease, pathogens, pests, parasites & predators of the taxa • existing regulatory mechanisms to protect the taxa • degree, magnitude, location & immediacy of threats to the taxa • taxonomic distinctiveness
IUCN Red List Categories & Criteria (International Union for the Conservation of Nature 2019)	• taxa's population size, trends & distribution • fluctuations in the extent, occupancy, number of populations, or number of mature individuals in the taxa • quantitative analysis of taxa's extinction probability
Managing Protected Areas, A Global Guide (De Lacy et al. 2006)	• species inventories • population size, trends & distribution of the taxa • habitat types & current conditions • degree, magnitude, location & immediacy of threats to the taxa • environmental services provided by the taxa

Table 1.4: Recommended mycorrhizal action items, and mycorrhizal implementation tips for natural areas. Expanded versions with references available in Supplement S7.

Mycorrhizal action items for natural areas

1. Manage areas with less historical disturbance for mycorrhizal fungi too.

2. Commit to restoring diverse, plant provenance and site appropriate, native mycorrhizal communities.

3. Develop mycorrhizal restoration plans alongside planting and natural regeneration plans.

Mycorrhizal implementation tips for natural areas

1. Native mycorrhizal fungi meaningfully contribute to ecosystem services and management goals.

2. Within and between species diversity of mycorrhizal fungi matters to outcomes.

3. Native mycorrhizal communities are depleted and degraded by a variety of disturbances.

4. Fully functional, diverse native mycorrhizal communities often do not regenerate quickly.

5. The needed native mycorrhizal communities are generally not present in sufficient numbers, combinations, or diversity on the plant material used in restoration.

6. Inoculation of plantings with native mycorrhizae optimized to plant provenance and site conditions boosts restoration outcomes.

7. Mass-produced mycorrhizal products can inhibit native mycorrhizae and yield poor results.

8. Inappropriate plant / soil / mycorrhizal pairings and poor timing can lead to poor results.

9. Optimal results are obtained with diverse mixes of native mycorrhizal communities appropriate to plant provenance and site.

10. Successful inoculation requires direct contact with live mycorrhizae or activated spores.
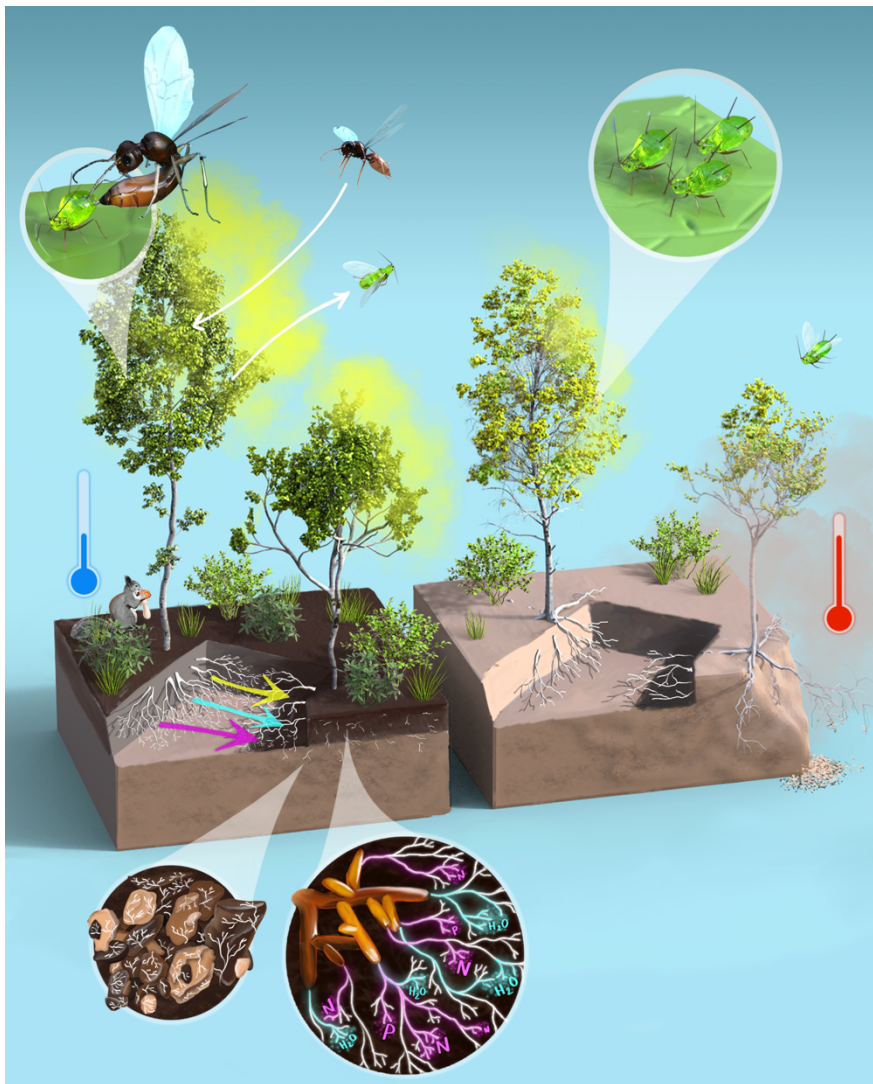
Figure 1.1: Examples of ecosystem services provided by mycorrhizal fungi (left) and results of their depletion (right), including: soil aggregation preventing erosion and dust; nutrient and water mining improving plant nutrition, diversity, and resilience; increasing food for small mammals and insects; and common mycorrhizal networks transporting nutrients, water, and pest warning signals; the resulting healthier, more robust community; increases in carbon storage; and reductions in temperature. Illustration by Kara Gibson and Victor Leshyk.
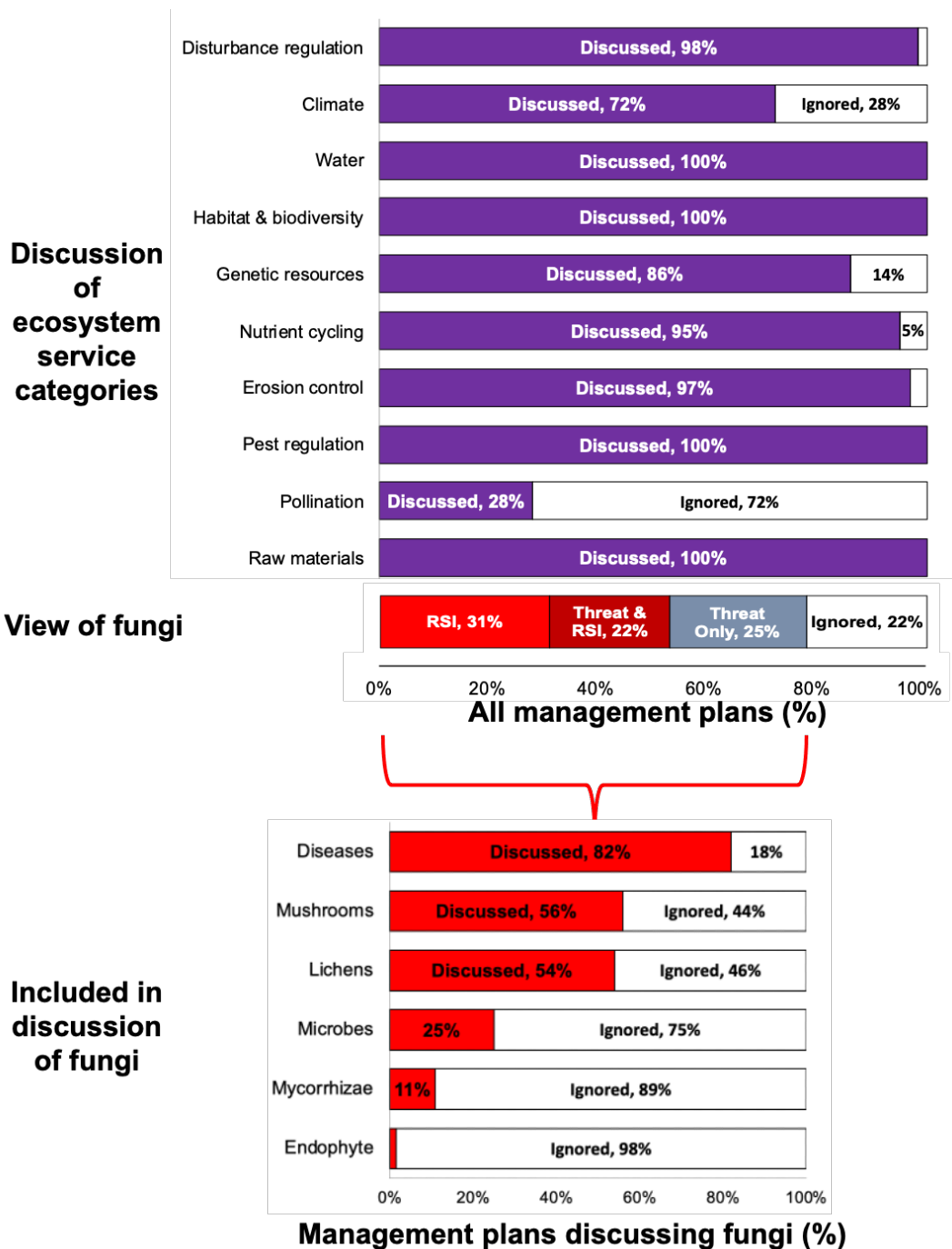
Figure 1.2: Results of systematically searching 130 management plans (United States Forest Service 2019; National Association of State Foresters 2019) for concern with individual ecosystem service categories and mycorrhizae (using terms found in Tables 1 and 2). Plans were largely concerned with the same ecosystem service categories to which mycorrhizae contribute (top graph). However, 46% of plans ignored the Kingdom of Fungi altogether or viewed them only as a threat (middle graph). Few plans mentioned mycorrhizae (8% of plans overall, and 11% of plans that mentioned fungi; bottom graph).

Figure 1.3: Relative representation of organismal groups within the estimated taxonomic diversity of life on earth (Larsen et al. 2017), international (International Union for the Conservation of Nature 2019; IUCN) and U.S. (United States Fish and Wildlife Service 2019a; ESA) conservation listings and fiscal year 2018 US federal research funding for research involving organisms (Star Metrics 2021). All organismal groups are represented in each series, but the representation of some groups is so small that they are not visible. Despite comprising 7.4% of the biodiversity on earth, fungi represent less than 0.1% of World Conservation Union and ESA listings. Although fungi appear in 5% of 2018 US federal research funding on organisms, 70% of this funding targeted disease. Supplement S3 and Tables S1 through S4 contain additional details of funding searches. Icons gratefully sourced from thenounproject.com.
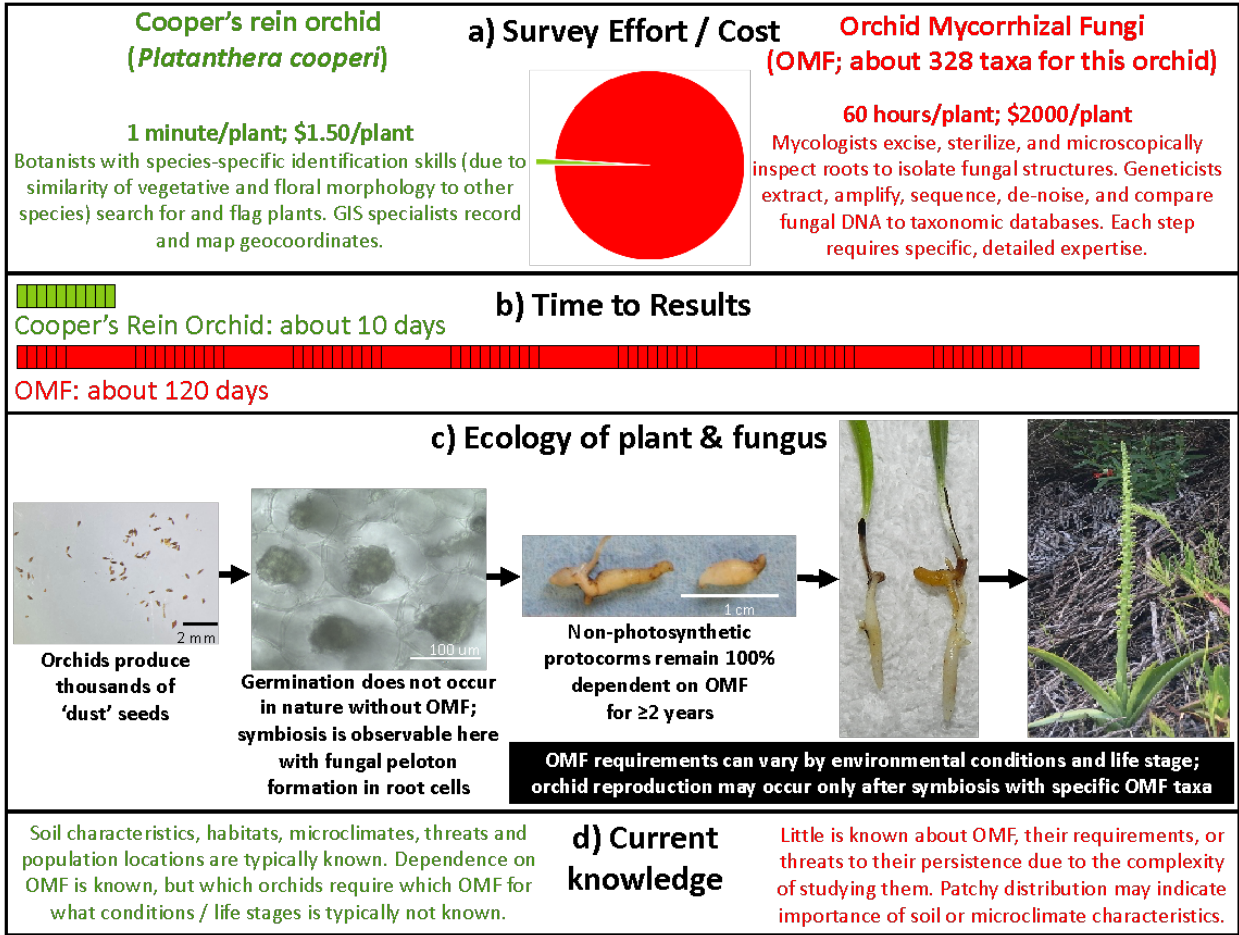
Figure 1.4: A case study comparing a) the relative effort and cost to survey and b) time to obtain survey results for a rare herbaceous plant and the beneficial orchid mycorrhizal fungi (OMF) upon which it obligately depends. Investigations of orchids reveal their c) obligate dependence on OMF, yet d) relatively little is known about OMF requirements concurrent with research cost and effort disparities. For additional details, see Supplement S4. Photos provided by Jyotsna Sharma.

Chapter 2:

EMF and AMF colonization after planting: implications for restoration and assisted migration during the UN's decade on ecosystem restoration

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Abstract

Many factors deplete and shift the mycorrhizal fungal communities upon which native plants depend, and consequently their often sizable and meaningful benefits to plant survival and growth, and a variety of ecosystem services. Yet, as the UN Decade on Ecosystem Restoration begins, active restoration of native mycorrhizal fungal communities is often overlooked as a strategy to enhance and sustain ecosystem restoration. Few studies focus specifically on implementation questions, such as the success and timeliness of natural mycorrhizal regeneration in disturbed soils and its effect on replanting success. Simultaneously, assisted migration of plant species or provenances has been suggested to hasten ecosystem climate change adaptations, but the consequences of assisted migration for mycorrhizal regeneration and associations remain poorly described. To determine the success of natural mycorrhizal regeneration, its interaction with plant provenance, and the consequences for restoration success, we evaluated naturally regenerating mycorrhizal colonization of *Populus fremontii* (Fremont cottonwood), a foundation riparian tree, in trees from one local and two assisted migrant source populations two years after planting. Three main lessons emerged. 1) Ectomycorrhizal colonization rates two years post-planting were considerably lower than those found in source populations, even under ideal conditions with an adjacent source of mycorrhizal host trees. 2) Within the planting site, proportions of EMF root tips and AMF arbuscules closely followed plant provenance, with trees from greater distances demonstrating fewer EMF root tips and AMF arbuscules. 3) For the plant provenance struggling the most, a close association between tree survival rates and mycorrhizal colonization was found. Results suggest that active mycorrhizal restoration and optimization of pairings between fungal, plant, and site provenances could maximize investments in restoration and natural area management, and help offset challenges posed by shortages in planting material and climate change.

Keywords: mycorrhiza, restoration, ecosystem restoration, riparian, cottonwood

Implications

- Natural regeneration of ectomycorrhizal fungi symbiotic with plant roots failed to meaningfully provide plants with these symbioses in the near term (2 years post-planting) after a legacy of agriculture.
- This was true despite ideal conditions for mycorrhizal spread: an adjacent source population with high mycorrhizal colonization rates and the use of water from that source population for irrigating the planted area.
- Trees from more distant source populations had fewer EMF root tips and AMF arbuscules for exchange between mycorrhizal fungi and plants.
- For trees from the population struggling the most at the site, even relatively small increases in mycorrhizal colonization were associated with increases in survival rates.
- Results indicate that management of natural areas and replanting investments would benefit from active restoration of symbiotic mycorrhizal fungi tailored to fungal, plant, and site provenances.

Introduction

As the UN Decade on Ecosystem Restoration begins, studies suggest that, in addition to the need for carbon sequestration via restoration, climate-related events such as increased wildfire incidence, size, and severity are amplifying the need for restoration and regeneration of natural ecosystems (Parks & Abatzoglou 2020; National Academies of Sciences, Engineering, and Medicine 2020; Fargione et al. 2021). However, seed and planting material resources have been declining in recent years, limiting the ability to scale-up restoration activities. Additionally, restoration benefits are constrained by limited post-planting activities and whether planting materials available are adapted to regional and local site conditions (Wheeler et al. 2015; National Academies of Sciences, Engineering, and Medicine 2020; Fargione et al. 2021).

In addition to these constraints, climate change presents a new set of challenges to ecosystem restoration. For example, climate changes may outpace the ability of plants to migrate via natural dispersal, such that the practice of using local seed stock and planting material for restoration may not continue to be the optimal strategy under climate change (Whitham et al. 2020). As the climate warms, the assisted migration of plant species or provenances from warmer locations may offer ecosystems a route to swifter adaptation (Etterson et al. 2020; Gomory et al. 2020; Saenz-Romero et al. 2021). However, assisted migration may increase disease risk, and assisted migrant plants frequently still have lower survival than locals (Tiscar et al. 2018; Simler et al. 2019; Cooper et al. 2019). For example, assisted migrant Fremont cottonwoods (*Populus fremontii* Sarg.) show at least 30% lower survival than locally sourced trees (Grady et al. 2011; Cooper et al. 2019). This seems to be due to the traits and adaptations of genotypes, populations and ecotypes (Ikeda et al. 2017; Cooper et al. 2019; Blasini et al. 2021). Consequently, ecotype adaptations can be an important consideration for sourcing planting material. Even local and regional ecotype adaptations to frost and cold can be an important

consideration for assisted migrants under climate change, due factors including the increased

temperature variability associated with climate change (Montwe et al. 2018). Cottonwoods, for

example, have developed contrasting adaptive strategies, known as adaptive trait syndromes.

Cottonwoods from the warmer Sonoran Desert (SD) ecoregion have developed physiological

traits that promote water movement through the plant for evaporative cooling which could risk

increased mortality under drought. In contrast, cottonwoods from the cooler Mogollan Rim (MR)

ecoregion have developed traits which reduce tree susceptibility to frost that could be helpful

under drought conditions but prove riskier in relation to warming (Hultine et al. 2020; Blasini et

al. 2021; 2022).

One strategy to improve restoration outcomes, stretch the impact of available planting

material, and help assisted migrant plants adapt to new sites is the active restoration of diverse

native mycorrhizal fungal communities. This strategy is used after disturbances known to

negatively impact mycorrhizal symbioses, such as pollution deposition, land use changes,

invasion by exotic species, and pesticide application (e.g. Egerton-Warburton & Allen 2000;

Meinhardt & Gehring 2012; Helander et al. 2018). This method is now suggested by multiple

studies and meta-analyses to promote survival, growth, and diversity of plant communities if

thoughtfully incorporated into restoration planning in conjunction with plant provenances,

palettes and site conditions (e.g. Wubs et al. 2016; Rua et al. 2016; Koziol and Bever 2017). At

least one meta-analysis has shown that the sizable benefits seen also improve with time since

planting (Neuenkamp et al. 2019). These findings concur with broader science reflecting that the

ability of organisms to survive and adapt is likely dependent on the confluence of their own traits

and adaptations with those of their microbiome (Zilber-Rosenberg & Rosenberg 2008;

Bordenstein and Thelis 2015; Whitham et al. 2020a). In fact, because of their contributions,

shorter life-cycles, and prolific genetic sharing, microbiota like mycorrhizal fungi may be

particularly crucial to promoting swift adaptation to change and rehabilitation of degraded systems (Wilkinson & Dickinson 1995; Coban et al. 2022).

Factors such as timing, plant species and plant provenance, and other site characteristics have proven integral in achieving optimal results (e.g. Johnson et al. 1992; Maltz and Treseder 2015; Mortimer et al. 2005). Neutral to negative effects can be found when these factors are not adequately addressed, or when plant roots fail to gain direct contact with live mycorrhizal fungal roots, or mass-produced products are utilized (Maltz and Treseder 2015; Rua et al. 2016; Saloman et al. 2022).

Despite the notable benefits seen from active restoration of native mycorrhizal fungal communities, many questions about the application of this method still exist. For example, it is relatively unknown how long natural regeneration of native mycorrhizal communities is likely to take. Thus, the costs to plant survival and growth, carbon sequestration rates, and ecosystem functioning without active restoration of native mycorrhizal communities cannot be calculated. To our knowledge, there is only one study that specifically investigated the amount of time required for the return of native mycorrhizal communities to a site after they have been depleted (Pankova et al. 2018). This study showed the negative effect of fungicide on plant cover, species composition, diversity, and mycorrhizal inoculation potential of the soil persisted at least five years after a single fungicide application. This study specifically addressed arbuscular mycorrhizal fungi (AMF) after a fungicide application, and we could find no studies investigating whether this varies by disturbance type, site conditions, plant provenance or type of mycorrhizal symbiosis (for example, with AMF or ectomycorrhizal fungi, EMF).

Similarly, we found few studies investigating the mycorrhizal fungal communities hosted by planting material from greenhouses and nurseries, and how these compare to mycorrhizal fungal communities in natural populations. This topic seems crucial to practical applications of

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mycorrhizal restoration, since different mycorrhizal fungal communities, or a lack of them, on planting material would explain the need for the additional, intentional step of mycorrhizal restoration. We found two studies focused on this issue, both demonstrating that although plants in greenhouses had mycorrhizal communities, they were less diverse and different than the mycorrhizal taxa found on plants growing in natural populations (Sykorova et al. 2007; Southworth et al. 2009). This is not surprising since symbiosis outcomes can change with every change in plant host, fungal partner, and environment (Rillig & Mummey 2006). However, due to the paucity of studies on this topic, it is unclear whether factors such as plant material type, nursery conditions, the inoculum handling techniques employed, or the type of mycorrhizal fungi (since AMF are thought to be more generalist than EMF, Maltz and Treseder 2015) impact the presence or suitability of mycorrhizal fungal communities on planting stock for restoration.

Additionally, there is an urgent need for large-scale, multi-species experimental studies to provide evidence that assisted migration is advisable given local adaptation of plants to other aspects of the abiotic environment besides temperature, and to address implications for interspecies interactions (Bucharova 2017). A few studies highlight the importance of interactions between, and local co-adaptation of plant, fungi, and soil and all emphasize the need to consider mycorrhizal symbioses and provenances in conjunction with plant provenances (Johnson et al. 2010; 2014, Rua et al. 2016). Yet, inter-species interactions are largely absent in studies of assisted migration (e.g. Etterson et al. 2020; Cooper et al. 2021; Saenz-Romero et al. 2021).

To address these knowledge gaps, we investigated how long it takes native mycorrhizal fungal communities to colonize and benefit replanted areas under ideal conditions, and the impact of assisted migration. Filling these knowledge gaps can inform the cost/benefit calculations for implementing active restoration of native mycorrhizal communities when it is

needed (for example, see Hart et al. 2017 Figure 4), and assist in the design of inoculum and plant provenance combinations that best harness biotic co-adaptation and site conditions.

We used provenance trials with assisted migration to replant a riparian corridor after over 50 years of agricultural legacy to investigate some of these questions. We utilized Fremont cottonwoods, a foundation riparian tree species. Riparian areas in the southwestern United States support 60-75% of the wildlife with less than 2% of the land area (Poff et al. 2012), and have suffered extreme losses (at least 90%, Zaimes 2007). Fremont cottonwood is one of the relatively few plant species to associate with both ectomycorrhizal and arbuscular fungi throughout their life cycles (EMF and AMF, respectively). Utilizing the dual colonization status and information on known cottonwood ecotypes, we investigated whether mycorrhizal fungal communities in adjacent habitat remnants had colonized planted areas two years post-planting under ideal conditions for mycorrhizal regeneration. We further investigated whether establishment of mycorrhizal associations would differ by tree provenance and ecotype, and assessed the importance of the mycorrhizal results to growth and survival. Although studies suggest that appropriate mycorrhizal inoculation is beneficial and needed after land remediation from agriculture (e.g. Wubs et al. 2016), we expected that if passive recruitment of mycorrhizal fungal colonization in planted trees was possible in the near term after disturbance, the conditions at our experimental site would provide relatively ideal conditions for this to occur. A natural source population was adjacent to and upriver from the site, and river water flowing through the natural population was used to irrigate the site, suggesting the dispersal of mycorrhizae from the adjacent natural population might be possible or even probable.

Thus, we hypothesized that 1) mycorrhizal colonization of replanted trees two years post-planting would be comparable to the adjacent natural source population; 2) if differences did exist between the natural population and the adjacent planted area, EMF colonization rates would

be more affected by the historical agriculture because AMF are more commonly associated with agricultural plants (Maltz and Treseder 2015; Hart et al. 2017); 3) colonization of planted trees would be highest for trees sourced from the adjacent natural population and lowest for trees sourced from the contrasting ecotype, based on studies reflecting the importance of plant, soil and mycorrhizal provenances and contrasting cottonwood ecotype adaptive trait syndromes (e.g. Johnson et al. 2010; Blasini et al. 2021; 2022); 4) plant survival and growth in the planted area would be associated with rates of EMF and AMF colonization, based on studies reflecting the benefits of mycorrhizal fungi and their restoration after disturbance (Wubs et al. 2016; Neuenkamp et al. 2019). The answers to these questions are important to both the fundamental scientific understanding of multi-species interactions and to restoration applications.

Methods

Source material and study sites

The Agua Fria common garden planting site (34.259688, -112.057859) comprises 1.2 Ha at Arizona Game and Fish's Horseshoe Ranch within the Agua Fria National Monument. The parcel on which the garden was planted has an agricultural legacy of at least 50 years (Cornerstone Environmental 2015). The site is near the climatic and elevational mid-point of Fremont cottonwoods. Cuttings from 16 populations across the climatic gradient of Fremont cottonwoods within Arizona were collected from trees at least 20 meters apart during the 2013-2014 winter, propagated in the greenhouse, and planted at the Agua Fria common garden site when they were approximately one year old, in October 2014. Replicates of genotypes were planted randomly within population plots in random order within a block. Blocks of population plots were replicated four times. During the growing season, trees were drip-irrigated with river water that first flowed through the adjacent Agua Fria natural population (34.257657, -

112.064022). The Gila soils series dominates the common garden replanting area with 39% sand, 37% silt, and 24% clay (USDA Natural Resources Conservation Service).

Trees within the common garden sampled for this study were sourced from three contrasting natural populations: 1) the adjacent Agua Fria natural population (34.257657, -112.064022) that experiences similar climatic conditions, the cooler Jack Rabbit natural population (34.989867, -110.622894), and the warmer Cibola natural population (33.360770, -114.698560). These source populations represent two distinct ecotypes (Blasini et al. 2021). The Jack Rabbit population is part of the Mogollon Rim (MR) ecotype with an adaptive trait syndrome that limits frost damage (and potentially reduces drought risk). The Agua Fria and Cibola populations are part of the Sonoran Desert (SD) ecotype with traits and phenology that maximize hydraulic efficiency and evaporative cooling, which may pose challenges under droughts of increasing frequency or severity (Ikeda et al. 2017, Blasini et al. 2021; 2022). Photos and locations of the natural populations and the common garden are depicted in Figure 2.1.

To compare mycorrhizal colonization between the common garden and natural stands of cottonwood trees, a subset of living trees in the common garden from the three natural source populations (Agua Fria, Jack Rabbit, and Cibola) were compared to a subset of trees located within two of those same natural populations (Agua Fria and Jack Rabbit). Trees in the common garden were approximately three years of age (two-years after planting in the field) and approximately 2m tall, at the time of sampling, while trees located within the natural populations were of an unknown age and at least 6 m tall. To help evaluate the impact of years since planting and tree age on results, trees planted within an area impacted by similar history to the common garden, and immediately adjacent to the common garden (the ranch house lawn), were also sampled. At the time of sampling, trees in the ranch house lawn area were at least thirty years of age and at least 6m tall, similar in size to trees located in the natural populations. None of the

trees were inoculated with mycorrhizal fungi. Soils in the adjacent Agua Fria natural population

are from the Gila soil series, relatively similar to those within the planting area. Soils in both of

the more distantly located natural populations (Jack Rabbit and Cibola) contained a higher

percentages of sand (71%, and 81%, respectively; USDA Natural Resources Conservation

Service). Photos and relative placement of sampling locations within Agua Fria National

Monument and Agua Fria are depicted in Figure 2.2. Climate and soil data for all sites is

presented in Table 2.1. The ecotypes (Sonoran Desert (SD) in pink and red, and Mogollon Rim

(MR) in blue), and the difference in mean annual temperature (MAT) between the natural

populations from which trees were sourced and where they were planted (MAT transfer distance)

is utilized in graphs showing differences between the three provenances within the common

garden to depict the change in climate that trees experienced (similar to the use of DD5 in

Cooper et al. 2019 and MAT in Blasini et al. 2022, for easy comparison).

Survival and growth measurements for planted trees

Survival and diameter at root crown for trees planted in the Agua Fria common garden

were sampled in January 2016 (year 1) and December 2016 (year 2). Calipers were used to

measure diameter at root collar (DRC), and area at root collar (ARC) was calculated from DRC

($ARC = 2\pi r^2$ where r = DRC/2). For survival, we focus on trees from the same garden blocks,

populations, genotypes during the same time-frame roots were sampled (year 2, n=134. To gauge

the best metric to use for growth, we harvested 26 trees (that were not part of the root sampling)

in September 2016 from cold, central, and hot populations within the Agua Fria common garden.

Trees were harvested manually at ground level and divided into stem, branches and foliage,

oven-dried to constant weight at 70°C for a period of 48 hours and weighed using an analytical

balance. Wood biomass and foliar biomass were measured by summing the weight of woody and

foliar components. ARC was found to be linearly correlated to aboveground biomass without the

need for additional transformations. For this reason, growth is calculated as the standardized difference in ARC during the same time period when roots were sampled for living trees from the same garden blocks, populations, and genotypes ((ARC for year 2 – ARC for year 1)/ ARC for year 1); n = 109).

Root sampling

During the autumn of 2016, fine roots within the dripline of each cottonwood tree in the root study were gathered from the four cardinal directions and traced from larger roots radiating from the base of each tree, placed into plastic bags, and frozen. To investigate hypotheses surrounding how plant provenance would affect colonization, three to six replicates of at least four genotypes from each of the three source populations in the common garden were utilized in this study (n=65). To provide reference colonization rates for cottonwood trees still growing in the same natural populations from which common garden trees were sourced, and in older trees planted in a similarly impacted area to the garden, roots were sampled in the same manner from cottonwood trees in the adjacent Agua Fria and distant Jack Rabbit natural populations (n=8), and the adjacent ranch lawn (n=3). Roots were immediately stored on ice, and frozen at -20°C within six hours of collection.

Colonization surveys

Roots from all samples were washed in tap water five times to remove debris. Remaining soil clumps firmly attached to the roots were not removed due to prior experience sectioning these types of clumps revealing that these can be dense clumps of EMF root tips and hyphae. Roots samples were frozen for preservation until they could be treated and evaluated individually.

EMF root tips were visually classified and counted under a dissecting microscope using the gridline intersection method (Brundrett et al. 1996). Over 7700 gridline intersections were

evaluated for 76 trees, and 1226 root tips were visually categorized and counted. Representative root tips for each morphotype were placed in extraction tubes for DNA sequencing.

Separate root subsamples were cleared and stained, and AMF hyphae, vesicles, and arbuscules classified and counted using the gridline intersection method under a compound light microscope (Brundrett et al. 1996). Care was taken to correctly identify dark septate endophytes separately, as indicated by septate hyphae. Over 7600 gridline intersections were evaluated for AMF colonization for 76 trees, and 6253 fungal organs were categorized and counted.

Low mycorrhizal colonization in the common garden, where the majority of samples were collected to address plant provenance questions, resulted in a limited ability to provide EMF community data for a small subset of the samples.

Mycorrhizal taxonomic identifications

A modified high molecular weight protocol (Mayjonade et al. 2016) was used to extract DNA from EMF root tips representative of the morphotypes seen. Amplification of a portion of the 18S region, all of ITS 1, all of the 5.8S region, all of the ITS 2 region, and a portion of the LSU was accomplished with the custom primers ITS1F_xt (5' - 3': CTTGGTCATTTAGAGGAAGTAAAAGTCGTAA, developed by Ron Deckert) and ITS4_fun (5` to 3`: AGCCTCCGCTTATTGATATGCTTAART, Taylor D.L. et al. 2016). ITS1F_xt was developed to avoid undesirable bacterial amplification by extending the primer sequence to include extra nucleotides found in the fungal 18S region that are not found in the prokaryotic 16S area (Gardes & Bruns 1993). The PCR product was cleaned (Rohland and Reich 2012), and amplification was conducted using Phusion Green Hot Start II High-Fidelity PCR Master Mix (ThermoFisher, Waltham, MA, USA) in the thermocycler (95°C for 2 min; 40 cycles of 30 s at 95°C, 45 s at 62°C, 75 s at 68°C; final extension of 5 min at 72°C). PCR products were examined using gel electrophoresis. Samples were then sequenced in the forward and reverse

direction using an ABI 3730 Genetic Analyzer at Northern Arizona University's Genetics Core Facility. Consensus sequences were created from Gap4 and preGap4 shotgun assembly (the Staden package, Bonfield et al. 1995). Sequences were aligned with known fungal sequences with BioEdit (Hall, 1999). Sequences under 500 base pairs in length were not used. To delineate operational taxonomic units (OTUs), the resulting sequences were identified using Ribosomal Database Project (RDP) Bayesian Classifier (Wang et al. 2007). Sequences were classified with two fungal ITS training sets. Sequences without a quality match were filtered-out of the dataset.

Soil Samples

The ribbon test and pH measurements were conducted at Northern Arizona University on soil samples from the common garden (n=6) and two natural populations, Agua Fria and Jack Rabbit (n=2 and n=3, respectively). Subsamples for pH were oven dried at 105°C and sieved (<2mm). Measurements of pH were taken on a 1:1 soil to water ratio using a Eutech Instruments 510 series bench pH meter with automatic temperature compensation (comparable to USDA soil survey methods). The ribbon test was conducted in accordance with the USDA protocol (Thien 1979).

Statistical analyses

All statistical analyses were conducted in R version 4.0.3 (R Core Team 2020). Individual trees were used as the independent experimental unit since plants were randomly planted across the garden, environmental heterogeneity was relatively low within the garden (Grady et al. 2011), and in exploratory data analyses both genotype and garden block failed to add significantly to statistical models. Bartlett's test for equal variance (also sensitive to non-normal distributions; McDonald 2014) was assessed using the bartlett.test() function in base R. Multiple variables failed this test. In particular, EMF colonization across the natural populations, garden, and ranch house (Bartlett's χ^2 = 40.1, df = 2, p <0.001), EMF colonization in the garden

(Bartlett's χ^2 = 40.047, df = 2, p < 0.001), AMF colonization in garden (Bartlett's χ^2= 5.3, df = 2, p < 0.1), and growth among populations in the garden (Bartlett's χ^2 = 56.3, df = 2, p < 0.001) failed Bartlett's test. In consideration for small sample sizes, non-normality and unequal variances, overall differences in colonization and growth were analyzed with Welch's ANOVA using the oneway.test() function in base R with the var.equal=FALSE setting, and post-hoc pairwise comparisons were made using the Tukey-Kramer test using the TukeyHSD () function in base R (McDonald 2014). Since our sample size was below 1000 (McDonald 2014), we utilized Fisher's exact test in base R using with the fisher_test() function in the rstatix library (Kassambara 2013) to test if survival differed among population groups in the garden. Post-hoc Fisher's exact tests with Bonferroni adjustments for multiple tests were used to test pairwise comparisons using the pairwise_fisher_test() function in the rstatix library.

To investigate if the proportions of EMF root tips and AMF arbuscules were associated with survival, survival rates and median colonization (EMF plus AMF arbuscules) rates were summarized by garden block and genotype and compared. This was accomplished using both linear regression (the lm() function) and Spearman's rank correlation (cor.test() function with the method='spearman' setting) in base R.

Results

Colonization of planted trees and natural populations

EMF colonization of trees significantly differed among the natural population, ranch house lawn, and garden (F = 11.06, df = 2, 4.2, p < 0.05; Table 2.2 and Figure 2.3). Post-hoc pairwise comparisons revealed that colonization in the natural population was significantly higher than that in either the ranch house lawn or the common garden (p <0.01 and p<0.001, respectively), while higher colonization in the ranch house lawn than in the garden was

marginally significant ($p < 0.10$). Other colonization measures were not significantly different across the three environments (Table 2.2).

As depicted in Figure 2.3, the hypothesis (1) that mycorrhizal colonization of replanted trees two years post-planting would be comparable to the adjacent natural source population was not supported, while the hypothesis (2) that EMF would be more affected if there were differences was supported.

Colonization of trees from different populations within the garden

EMF and arbuscule colonization of trees in the garden differed across populations ($F = 15.31$, df = 2, 27.05, $p < 0.001$ and $F = 5.79$, df = 2, 30.98, $p < 0.01$, respectively). Post-hoc pairwise comparisons revealed that EMF and arbuscule colonization both differed significantly between groups ($p < 0.05$ and $p < 0.001$, respectively, Table 2.3 and Figure 2.4). DSE and overall AMF colonization did not differ significantly between populations in the garden (Table 2.3).

The hypothesis (3) that trees from more remote locations would show reduced colonization was supported for both EMF and AMF arbuscule colonization. However, differences were not particularly greater for trees from the contrasting ecoregion (MR). As shown in Figure 2.4, despite extremely low mycorrhizal colonization of trees in the common garden overall, the colonization of tree roots by EMF and AMF arbuscules was significantly less in trees from more remote source populations than in the local plant provenance, regardless of ecotype and adaptive trait syndrome.

Survival and growth of trees from different populations within the garden

Survival to year two differed significantly between populations ($p < 0.001$; Table 2.3), with post-hoc pairwise comparisons showing trees from Cibola demonstrated significantly lower

survival than Jack Rabbit or the local Agua Fria population (p < 0.001 for both; Table 2.3 and Figure 2.4).

Growth from year one to year two significantly differed between populations planted in the common garden (F = 6.80, df = 2, 163.33, p < 0.01). Post-hoc pairwise comparisons revealed that Jack Rabbit demonstrated significantly higher growth than either the local Agua Fria or the Cibola populations (p < 0.001 and < 0.01, respectively; Table 2.3 and Figure 2.4).

There was a significant and strong relationship between tree survival rates and median colonization rates (EMF + arbuscule; adjusted R^2 = 46%, F = 4.93, df = 5, 18, p < 0.01, and r_s= 48%, p <0.05, for linear regression and Spearman's rank correlation coefficient, respectively). As can be seen in Figure 2.5, the summary of the linear regression shows that the relationship between survival and colonization for the struggling trees from the warmer, assisted migrant Cibola population drives this relationship (t = -3.661, p <0.01 and t = 3.21, p < 0.01 for the population and population by colonization interaction, respectively).

The hypothesis (4) that mycorrhizal colonization would be significantly correlated with survival and growth, demonstrating the importance of the symbioses for restoration goals, was partially supported. For the population of trees struggling most in the common garden, mycorrhizal colonization was largely and significantly correlated with survival. Survival rates in trees from the other two source populations was universally high. Growth across tree source populations reflected the ability of cold populations to respond positively to warming seen in Cooper et al. (2019).

Mycorrhizal taxonomic identifications

Taxonomic identifications from natural population EMF root tips are shown in Table 2.4. Warcup results indicate that representative EMF colonized root tips from the two most abundant morphotypes in the root samples (DRKKNB and BLKFNG) were *Tricholoma populinum* and

Tomentella spp., respectively (Table 2.4). Other taxa represented by natural population morphotypes included *Amanita* spp. and *Sebacina* spp.

Discussion

In this study, ectomycorrhizal colonization of cottonwood trees two-years post planting after historical agriculture had not reached levels comparable to those in natural areas where trees were sourced from, either near or far. Despite this, trees in the planted area from the local plant provenance still showed significantly higher colonization by EMF and AMF arbuscules than trees from remote provenances. And despite this, trees from the population with the lowest levels of colonization and lowest survival rates, reflect a strong relationship between increasing colonization and higher survival. These results improve our understanding of fundamental principles of mycorrhizal symbioses and have urgent, practical implications for restoration and assisted migration,.

Natural recruitment and recovery of mycorrhizae after disturbance

This study contributes to the broader understanding of timelines for natural mycorrhizal recruitment and recovery, and our understanding of one reason why a separate step of restoring diverse native mycorrhizal fungal communities often has such beneficial impacts (Neuenkamp et al. 2019). Although there has been clear evidence for some time that incorporating a separate, intentional mycorrhizal restoration step into habitat regeneration is highly beneficial after certain disturbances, few studies have addressed the underlying reasons. The causes are particularly curious when the sources of disturbance are local and it might be otherwise assumed that mycorrhizal spores could disperse on wind and water to swiftly regenerate post-disturbance. To our knowledge only one study specifically addressed the length of time required for natural mycorrhizal recruitment and regeneration after a disturbance, and this study specifically addressed one type of disturbance and one type of mycorrhizal symbioses (fungicide application

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and AMF, respectively). Five years after fungicide application, plant cover and communities, and AMF mycorrhizal potential in the soil were still well below that for control plots (Pankova et al. 2018). We are aware of two other studies that touch on this subject, and the current study concurs with concepts presented in both studies. Peay et al. (2010) found decreases in ectomycorrhizal species diversity with increasing isolation of mycorrhizal tree islands, indicating that dispersal is likely a challenge for ectomycorrhizae. In a review of evidence from regenerating forest stands after clear-cut logging, Jones et al. (2003) cite multiple lines of evidence suggesting that living mycorrhizae on roots of immediately adjacent conspecific and allospecific plants, and their hyphae, are an important source of inoculum for, and support rapid colonization of, regenerating seedlings. Notably, this would be an important source of inoculum for natural regeneration and habitat restoration that would be lacking after many kinds of disturbances that reduce native plant hosts, or their immediate adjacency or density.

Our study took place under conditions that seemed ideal for the natural regeneration of native mycorrhizae (with an adjacent, natural population with high colonization rates that could provide a native inoculum source, and with water flowing through that source population prior to being used to irrigate the planted area). Despite this, EMF colonization was extremely low in planted trees two years after planting, even when trees had been originally sourced from the adjacent population, ruling out plant provenance as the main cause. While an argument could be made that reduced EMF colonization levels in the planted area were due to tree age, data from the at least 30 year old trees found in the similarly disturbed, adjacent ranch house lawn show EMF colonization that is marginally but significantly different from, and intermediate between the common garden trees and the natural population trees. This suggests that while tree age could have an effect, it is certainly not the only factor in colonization differences between the natural populations and the planted area. Gehring et al. (2006) found that when inoculum is available,

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cottonwood AMF and EMF colonization rates can be changed over the course of weeks in response to changes in environmental variables such as watering, and many greenhouse studies reflect higher rates of colonization with inoculation than seen in the common garden, even in relatively short duration studies (e.g. Meinhardt & Gehring 2012). Another potential cause of low EMF colonization rates in the planted area could be potentially elevated nutrient levels in the soil from fertilization during agriculture that preclude the need for mycorrhizal nutrient mining services. However, this seems unlikely since mycorrhizae are known to provide a variety of services besides nutrient mining (e.g. Markovchick et al. in review, Chapter 1), and since many studies show the benefits of mycorrhizal inoculation for plants during restoration after agriculture, even in systems where AMF are more predominant than the current study (such as prairie systems where many native plants utilize only AMF; crop plants tend to have AMF associations; e.g. Koziol & Bever 2017). In fact, cottonwoods from the same assisted migrant populations at the same common garden as in the current study, were shown to have higher colonization rates five years post-planting (Hultine et al. 2020). This timeline seems relatively congruent with findings from Pankova et al. (2018), and suggests that swift dispersal, even under the ideal conditions found in our study, may indeed be a limiting factor.

Implications for assisted migration and plants under stress

Despite low colonization in tree roots from the common garden overall, more locally sourced trees demonstrate significantly higher EMF and AMF arbuscule colonization than their counterparts from more remote locations. These findings are consistent with other literature, predominantly from AMF systems, demonstrating that plants and fungi are co-adapted to each other and site conditions (Johnson et al. 1992, 2010, 2014). In tandem with existing literature, our study suggests that even when native mycorrhizal fungi are available, they may be less compatible with assisted migrant plants. Crucially, though, the strong correlation we found

between survival rates and colonization rates in trees from the warmer assisted migrant population with the lowest survival rates demonstrates that for assisted migrants and plants struggling the most, even small increases in mycorrhizal symbioses can be associated with increased survival. This finding concurs with the broader concept suggested by the literature that mycorrhizal symbioses can be particularly important for plants under stress, regardless of the cause of stress (drought, disease, lack of nutrients, etc.; Miozzi et al. 2020; Bingham and Simard 2011; Sarkar et al. 2015), suggesting that as stressors increase under climate change, these symbioses may become increasingly important to plant survival.

Mycorrhizal colonization, ecotypes, and adaptive trait syndromes

Interestingly, the colonization and survival results in our study do not align with the idea that the most extreme differences would be found in trees from the contrasting ecotype or adaptive trait syndrome (Blasini et al. 2021; 2022). Instead, assisted migrant trees from the same ecotype as the common garden (the warmer Sonoran Desert (SD) ecoregion) had even lower rates of EMF colonization and survival than assisted migrant trees from the contrasting ecoregion as the common garden (the cooler Mogollon Rim (MR) ecoregion), despite the absolute difference in mean annual temperature for both types of assisted migrants being relatively similar. This finding suggests that differences in mycorrhizal symbioses may be operating at a different scale than physiological adaptive trait syndromes. For instance, primary differences in physiology may be determined at the regional level by climatic differences, followed by smaller differences at the population level (Blasini et al. 2021), while differences in ectomycorrhizal compatibility may be determined primarily by more local factors within individual populations or sites. This finding resembles the concept that tighter fidelity on ecological time scales would be necessary to consider hosts and microbiomes as holobionts (Douglas & Werren 2016). Indeed, if physiological traits are primarily determined by variation at

one scale while symbiotic compatibility or effectiveness is primarily affected by factors on a finer scale, the lack of fidelity on geographic scales between host and microbiome may provide an additional reason why hosts and microbiomes are better thought of as ecological communities rather than holobionts,

Implications for restoration and regeneration

The long timeline for mycorrhizal regeneration in the replanted area under ideal conditions suggests that, in addition to the known benefits of and need for mycorrhizal restoration (Wubs et al. 2016; Koziol & Bever 2017; Neuenkamp et al. 2019), the need for swifter regeneration and restoration after increased disturbances under climate change will require mycorrhizal restoration. The reduced mycorrhizal symbioses in trees from remote plant provenances, and strong relationship between survival and even slight increases in mycorrhizal colonization for struggling trees both suggest that restoration of diverse mixes of native mycorrhizae will become an increasingly important tool as environmental stressors increase and assisted migration of plant provenances becomes more common. Indeed, in concert with previous studies, the low colonization seen in assisted migrants in our study, coupled with the strong relationship between slight increases in colonization and increased survival rates of struggling migrants, suggests that inoculating assisted migrant plants with appropriate, diverse mixes of native mycorrhizal fungi could improve their chances at new locations. Additional research is needed to determine the optimal mixes of native mycorrhizal fungi for this purpose, given studies reflecting the co-adaptation of plants and fungi to each other and to site conditions; (Johnson et al. 1992; 2010; 2014), optimal mixes might include mycorrhizal fungi from the plant source populations (as in Remke et al. 2020), from the site itself or adjacent habitat remnants, or a mix of both.

Conclusions

Soil microbiota are often key to supporting crucial ecosystem services and swift adaptation (Wilkinson & Dickinson 1995; Coban et al. 2022). Mismatches in aboveground and belowground biodiversity and conservation needs, and reports of declines in mycorrhizal fungi due to a variety of disturbances highlight the need to consider mycorrhiza and microbiota in restoration, regeneration, and assisted migration (Meinhardt & Gehring 2012; Helander et al. 2018; Cameron et al. 2019). Indeed, findings from this study support the urgent need for incorporating large-scale consideration of interspecies interactions in assisted migration studies (Bucharova 2017). If the full diversity of the mycobiome and microbiome are not conserved across the landscape, restored and maintained, plants may not have their optimal partners (particularly beneficial when plants are exposed to stressors) in the future, having cascading effects on their ability to adapt to changing climates and circumstances. Maintaining and supporting the full diversity of the mycobiome and microbiome during regeneration and restoration activities could provide ecosystems with the highest chance of adapting at the pace of climate change and optimizing the services provided by remaining natural areas. This approach also has the advantage of being aligned with the goals and ethics of ecosystem restoration, and being consistent with United Nations' Ecosystem Restoration Principle Three (FAO et al. 2021).

Acknowledgements

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Tables and figures

Table 2.1: Climate and soil characteristics for natural source populations and common garden.

	Agua Fria Common Garden	Agua Fria Natural Population	Jack Rabbit Natural Population	Cibola Natural Population
Population Code	-	CAFAUG	JLAJAK	CCRCOL
Ecotype	-	SD	MR	SD
Elevation (m)	988	988	1507	70
Latitude	34.259688	34.257657	34.989867	33.36077
Longitude	-112.057859	-112.064022	-110.622894	-114.69856
Mean PPT (cm)	35	35	15.7	7.4
Max VPD (hPa)	18.4	18.4	27.6	34.1
MAT (°C)	18.4	18.4	14.2	22.1
MAT Transfer (°C)	-	0	4.2	-3.7
USDA Soil Series	Gila Soils	Barkerville Cobbly Sandy Loam	Ives Soils	Lagunita Loamy Sand
USDA Soil Content	39% sand, 37% silt, 24% clay	68% sand, 20% silt, 13% clay	71% sand, 17% silt, 13% clay	81% sand, 17% silt, 3% clay
USDA Soil pH	-	7	8.2	8.2
Sample pH Results	7.40 - 8.48	6.81 - 6.95	6.75 - 7.98	-
Sample Ribbon Test Results	Silt Loam ; Loam	Silt Loam; Loam; Loamy Sand	Silt Loam ; Silt Clay Loam	-

Notes
1) Climate data for 2016-2018 is from PRISM Climate Group (2020).
2) USDA soil data is from United States Department of Agriculture (USDA), Natural Resources Conservation Service (2020).
3) USDA pH values were not available for the common garden. Ribbon and pH tests were applied to a soil samples from the sites at Northern Arizona University as in the methods section. Roots and soil samples were not available for the Cibola natural population.
4) Climate abbreviations are as follows: mean annual temperature (MAT), precipitation (PPT), vapor pressure deficit (VPD).

Table 2.2: Medians, inter-quartile ranges, Welch's ANOVA results, and post-hoc Tukey-Kramer results for mycorrhizal measures in trees from three environments. Medians are shown under each location, with inter-quartile ranges in parentheses. Significant differences are in bold. Marginally significant differences are noted with an asterisk.

	a) Natural Populations	b) Ranch house	c) Common garden	Welch's ANOVA	Tukey-Kramer
n	8	3	65		
EMF (%)	**65.5 (21.7)**	**19.8 (26.9)**	**4.0 (4.0)**	**F = 11.06, df = 2, 4.2, p = 0.02**	a-b: p = 0.007 a-c: p = 0.00 b-c: p = 0.09*
AMF (%)	53.5 (33.5)	64.0 (18.5)	56.0 (29.0)	F = 0.93, df = 2, 4.5, p = 0.46	
Arbuscules (%)	1.0 (1.3)	0.0 (2.5)	1.0 (2.0)	F = 0.02, df = 2, 4.6, p = 0.97	
DSE (%)	7.0 (19.5)	16.0 (29.5)	21.0 (45.0)	F = 0.46, df = 2, 4.6, p = 0.66	

Table 2.3: Summary statistics and statistical test results for colonization (n=65) and survival and growth (n=134 and 109, respectively) for trees planted in the garden from three source populations. Significant differences are in bold.

	a) Jack Rabbit	b) Agua Fria	c) Cibola	Statistical Test (Overall Comparison)	Results (Overall Comparison)	Post-Hoc Test	Post-Hoc Results
Ecotype	MR	SD	SD				
MAT Transfer (°C)	4.2	0	-3.7				
n	31	15	19				
EMF (%) Median (IQR)	4.0 (9.1)	12.4 (8.0)	2.0 (4.0)	Welch's ANOVA	F = 15.31, df = 2, 27.052, p =0.000004	Tukey-Kramer	a-b: p = 0.04 a-c: p = 0.04 b-c: p = 0.0001
AMF (%) Median (IQR)	56.0 (23.5)	65.0 (25.5)	48.0 (35.0)	Welch's ANOVA	F = 1.18, df = 2 , 30.0, p = 0.32		
Arbuscules (%) Median (IQR)	0.0 (1.5)	4.0 (3.5)	0.0 (1.0)	Welch's ANOVA	F = 5.79, df = 2, 30.98, p = 0.007	Tukey-Kramer	a-b: p = 0.0009 a-c: p = 0.9 b-c: p = 0.0009
DSE (%) Median (IQR)	22.0 (37.5)	28.0 (45.0)	3.0 (50.5)	Welch's ANOVA	F = 0.39, df = 2, 29.94, p = 0.68		
Survival to Year 2 (Overall)	89.8%	85.7%	63.8%	Fisher's Exact Test, two-tailed	p-value = 0.0000004	Fisher's Exact Test, Bonferroni correction	a-b: not significant a-c: 0.000002 b-c: 0.0002
Growth to Year 2: Median (IQR)	3.29 (2.29)	2.78 (1.21)	2.42 (1.77)	Welch's ANOVA	F = 6.80, df = 2, 163.33, p = 0.0015	Tukey-Kramer	a-b: p = 0.0028 a-c: p = 0017 b-c: p = 0.94

Table 2.4: Operational taxonomic unit information and UNITE BLASTn matches.[1]

OTU information				Representative top hits from BLAST/UNITE searches					
Location	Morphotype Name	OTU name	BLAST Accession #	UNITE Reference	UNITE Description	UNITE Score	E-value	BLAST % Identity	Notes
Jack Rabbit Natural Population	BLKFNG	*Tomentella* 1		EF218826 SH1502204.08FU	Thelephoraceae	922	0	97.3	
				UDB013728 SH1502204.08FU	*Tomentella* spp.	918	0	97.1	
	BLKFNG	*Tomentella* 1		UDB002905 SH1502250.08FU	*Tomentella* spp.	985	0	98.7	
				UDB0757836 SH1544167.08FU	Agaricomycotina	723	0	89.8	
	ORGTUB	*Sebacina* 1		FJ803944 SH1513233.08FU	*Sebacina* spp.	1038	0	96.9	2
				MH038099 SH1513233.08FU	*Sebacina epigaea*	1033	0	97.1	
	WHTFNG	*Sebacina* 2		GU328501 SH1513233.08FU	*Sebacina* spp.	985	0	99.6	2
				KF000427 SH1561861.08FU	*Sebacina epigaea*	948	0	98.5	
Agua Fria Natural Population	DRKKNB	*Tricholoma* 1		KC146366 SH1520333.08FU	*Tricholoma populinum*	800	0	99.1	
	WHTMAS	*Amanita* 1		MN204485 SH1611339.08FU	*Amanita* spp.	1074	0	99	
				KP221307 SH1611313.08FU	*Amanita populiphila*	1018	0	98.7	

Notes
1) Sequence matches include the top fungal match to each OTU and, where informative and possible, additional named matches with high similarity.
2) *Sebacina* spp. are known to form ectomycorrhizae as well as ericoid and other mycorrhizae (Kariman et al. 2018).

Figure 2.1: Map and photos of the three natural populations across the climatic and elevational gradient from which trees in this study were sourced. Sonoran Desert ecotype locations, Cibola and Agua Fria are in shown in red and pink. The Mogollon Rim ecotype location is shown in blue. The mid-elevation location of Agua Fria is also the location of the Agua Fria common garden. Photos by Lisa Markovchick and Kevin Grady.
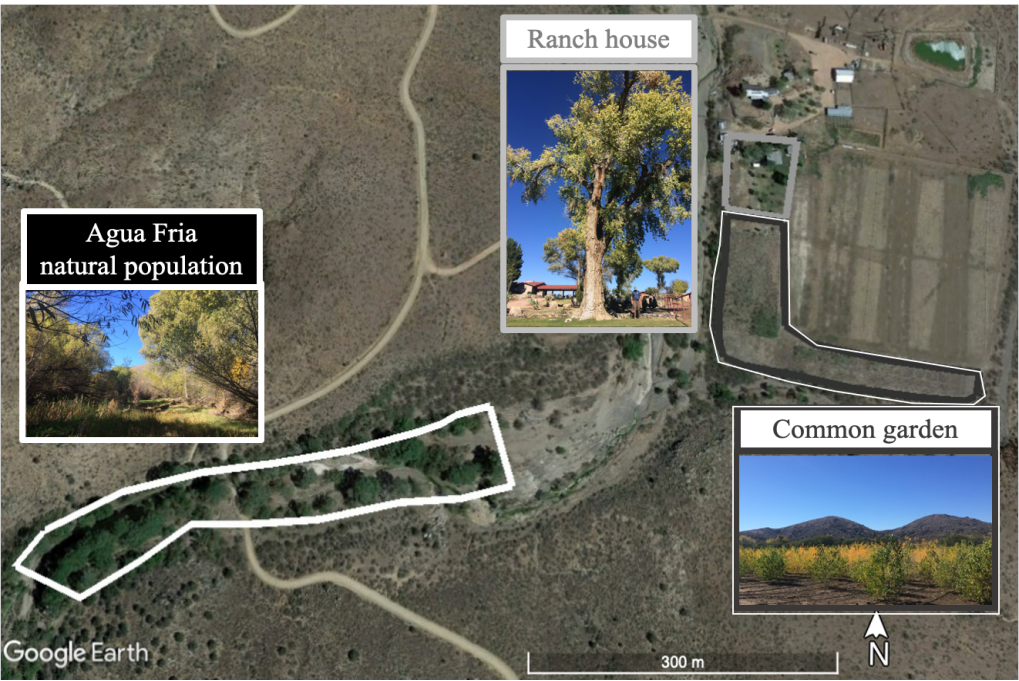
Figure 2.2: Map and photos of areas compared at Agua Fria: the Agua Fria natural population, the ranch house lawn with existing older trees, and common garden experiment. Color swatches next to photos indicate colors used in figures comparing trees from each location at Agua Fria. Photos taken by Lisa Markovchick.
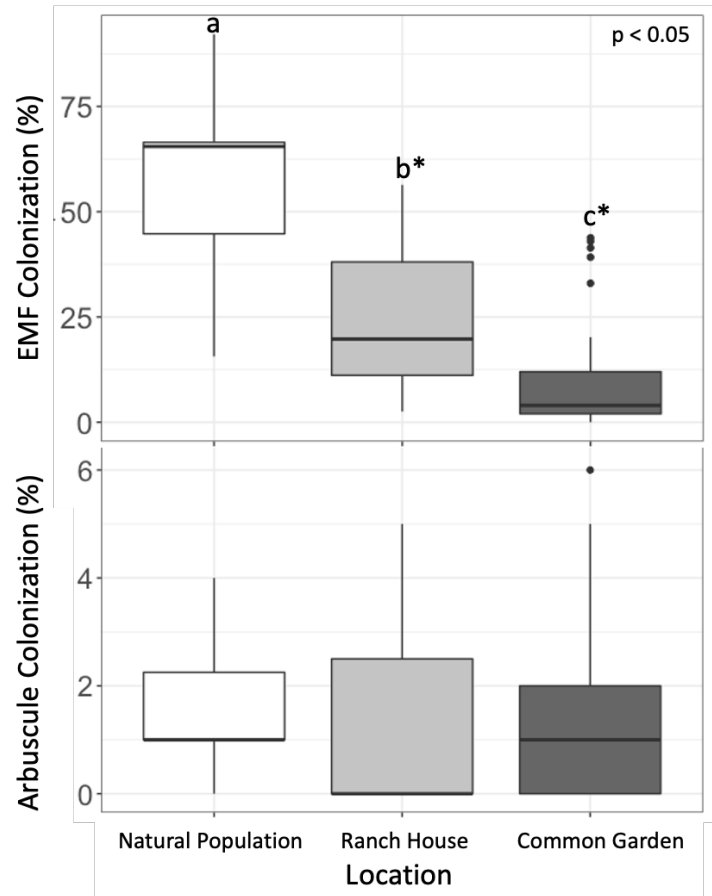
Figure 2.3: Box and whisker plots (left) showing medians and 25th and 75th percentiles for EMF colonization (top) and arbuscule colonization (bottom) in trees from the natural source populations, ranch house lawn, and the common garden. Differing letters across box and whisker plots indicate significant differences at α = 0.05, asterisks between pairs indicate significant differences at α = 0.10.
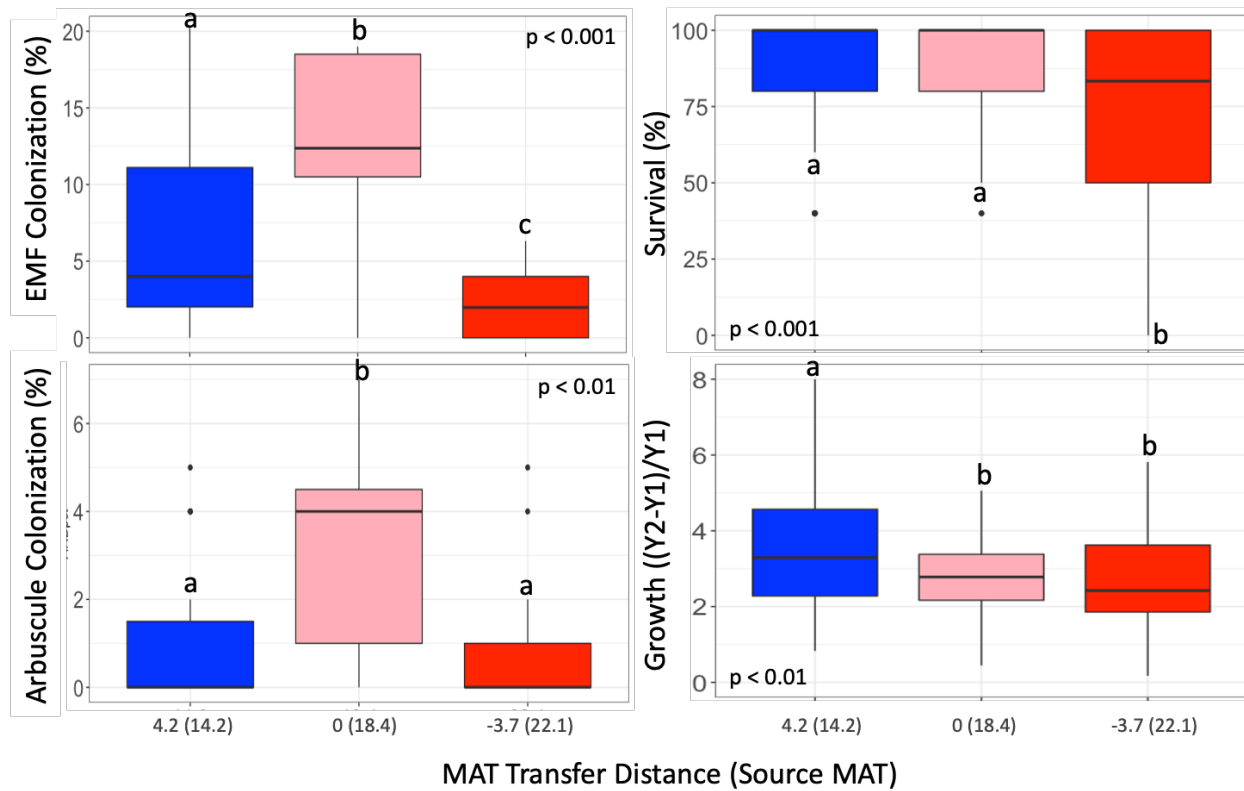
Figure 2.4: Common garden EMF colonization (top left), and presence of arbuscules (bottom left) of a subset of trees for which roots were sampled; population level survival through the second growing season, and growth of trees during the second growing season after planting (right) for trees sourced from three natural populations, presented by the MAT of the source populations and MAT transfer distance to the common garden. Populations in blue belong to the Mogollon Rim (MR) ecotype and adaptive syndrome described by Blasini et al. (2021), while the population in red belongs to the Sonoran Desert ecotype (SD) ecotype. Box and whisker plots show the median, 25th and 75th percentiles (boxes). Differing letters across source populations represent significant differences between populations.
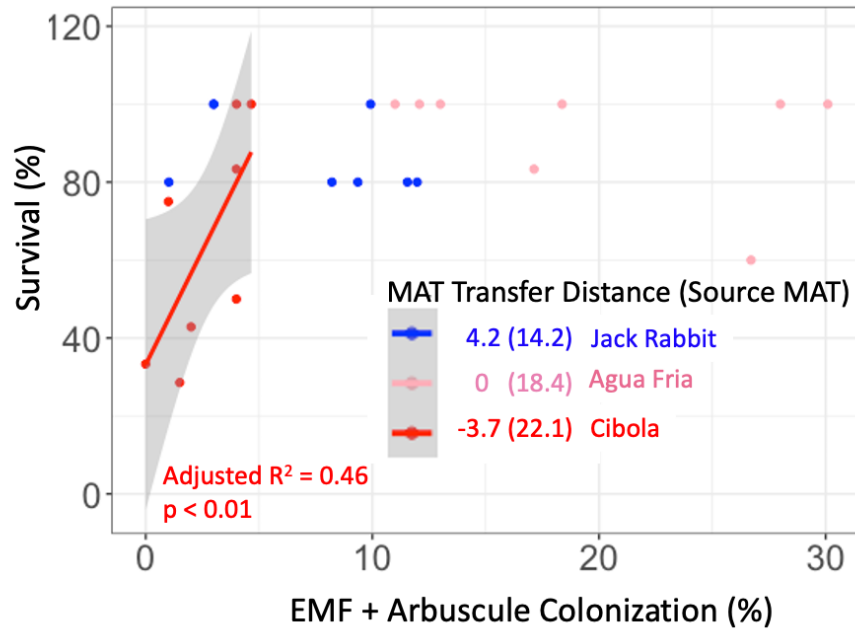
Figure 2.5: The significant, positive relationship between survival rates and median colonization rates, summarized by garden block and tree genotype, for the struggling Cibola population (in red), revealing that although colonization rates were often lowest in this population, even minimal colonization rates were associated with increased survival. The relationship was not significant for the other two populations, which already had quite high survival rates overall.

Chapter 3: Utilizing symbiotic relationships and assisted migration in restoration to cope with multiple stressors, and the afterlife of invasive species

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Abstract

Under climate change, the need for and importance of natural regeneration, replanting, and restoration is expected to grow while planting and recruitment success decrease, and planting material shortages continue to constrain replanting efforts. Thus, strategies to optimize restoration effectiveness seem increasingly important. Assisted migration and the restoration of native mycorrhizal communities appear promising. However, intraspecies assisted migrations often still show reduced survival compared to local provenances, and mycorrhizal inoculation, effective if well-matched to plants and site conditions, can have neutral to negative results with poor pairings. Few studies examine the interaction between these two strategies and invasive species soil legacies, particularly under drought conditions exacerbated by a warming, drying climate. To evaluate these interactions, we planted *Populus fremontii* (Fremont cottonwoods) from two ecoregions (local and warmer) in soils with and without legacies of invasion by *Tamarix* spp. (tamarisk), and with and without restoration of native mycorrhizal fungi. Four main lessons emerged. 1) The soil legacies left behind after tamarisk invasion reduced survival by 85%. 2) Actively restoring a diverse, native community of mycorrhizal fungi after tamarisk invasion doubled and then tripled survival during the first and second field seasons, respectively. 3) Inoculation sometimes had neutral and even negative effects, interacting with timing, pairing between tree and inoculum sources, and site conditions. 4) During the second field season, assisted migrants survived at twice the rate of trees from the local ecoregion, if inoculated with an appropriate and diverse native mix of mycorrhizal fungi. Results emphasize the detrimental effects of soil legacies left behind after tamarisk invasion, the beneficial effects of appropriate mycorrhizal restoration, and the need to thoughtfully optimize pairings between plants, fungi, and site conditions.

Keywords: assisted migration, mycorrhiza, restoration, ecotype, invasive species

Implications

- Soil legacies left by invasive tamarisk trees reduced cottonwood survival by 85%.
- The survival of native cottonwoods after tamarisk invasion doubled and tripled (during the first and second growing seasons), when trees were provided with an appropriate, diverse mix of symbiotic, native mycorrhizal fungi.
- During the second field season, assisted migrants survived at twice the rate of trees from the local ecoregion, if inoculated with appropriate mycorrhizal fungi.
- However, inoculation had varied effects (positive to negative) on the survival of native cottonwoods, when planted in soils without a tamarisk legacy, interacting with the pairing between plant and inoculum source, and timing.
- Results emphasize the distinct benefits of appropriately matched mycorrhizal and plant pairings, and the afterlife effects of invasive species.

Introduction

The need for and importance of natural regeneration, replanting, and restoration under climate change is expected to increase for many reasons as the UNs' Decade on Ecosystem Restoration progresses. In addition carbon sequestration needs, the increasing frequency, size and severity of natural disasters like wildfires will increase the need for the restoration and regeneration of ecosystems (Parks & Abatzoglou 2020; National Academies of Sciences, Engineering, and Medicine 2020; Fargione et al. 2021). However, planting material shortages are constraining restoration and restoration success (Wheeler et al. 2015; National Academies of Sciences, Engineering, and Medicine 2020; Fargione et al. 2021).

Additionally, climate changes may outpace the ability of plants to adapt or migrate via natural dispersal, such that the practice of using local seed stock and planting material for restoration may not continue to be optimal (Whitham et al. 2020). Infusing additional genetic diversity into ecosystems using strategic assisted migration of plant provenances, or species, from warmer or drier locations, may help ecosystems adapt to the warmer, drier climate at a pace on par with climate change (Etterson et al. 2020; Gomory et al. 2020; Saenz-Romero et al. 2021). However, assisted migrant plants are often more susceptible to disease and continue to show lower survival than local provenances (Tiscar et al. 2018; Simler et al. 2019; Cooper et al. 2019). For Fremont cottonwoods, survival and growth for trees from warmer plant provenances can be reduced by 30% (Grady et al. 2011; 2015; Cooper et al. 2019). The local suitability of Fremont cottonwoods for the shifting climate is affected by the traits and adaptations of their genotype, population, and ecotype (Ikeda et al. 2017; Cooper et al. 2019; Blasini et al. 2021; 2022). Trees from the warmer Sonoran Desert (SD) ecoregion have developed physiological traits which promote water movement through the plant for evaporative cooling (but may leave trees susceptible to drought-driven stress), while trees from the cooler Mogollon Rim (MR) ecoregion

have developed traits which reduce their susceptibility to frost (which may help with drought-resilience, but result in more susceptibility to heat stress; Hultine et al. 2020; Blasini et al. 2021; 2022). Consequently, regional and population level adaptations can be an important consideration for sourcing planting material, but the field efficacy of assisted migration, and how to optimize provenance selection, is not always clear. There is also an urgent need for large-scale, multi-species experimental studies to provide evidence that assisted migration is advisable given local adaptation of plants to other aspects of the abiotic environment besides temperature, and to address implications for interspecies interactions (Bucharova 2017).

This urgent need intersects with another strategy suggested to improve restoration outcomes, stretch the impact of available planting material, and help assisted migrant plants adapt to new sites, the active restoration of diverse native mycorrhizal fungal communities symbiotic with native plant roots. Many kinds of disturbances negatively impact mycorrhizal fungi, such as pollution deposition, land use changes, invasion by exotic species, and pesticide application (e.g. Egerton-Warburton & Allen 2000; Meinhardt & Gehring 2012; Helander et al. 2018). In particular, invasion by exotic plant species has been documented to simultaneously reduce native plant biomass, survival, and diversity in conjunction with reducing or shifting their mycorrhizal fungal partners. These types of results have been found with invasive plants including *Cynodon dactylon* (Bermuda grass), *Calamagrostis epigejos* (bush grass), *Avena barbata* (slender wild oats), *Bromus hordeaceus* (soft chess), *Bothriochloa bladhii* (old world bluestem), *Tamarix* spp. (tamarisk), and others (Hawkes et al. 2006; Wilson et al. 2012; Endresz et al. 2013). Decreases and shifts in mycorrhizal fungal communities from invasive species seem to arise from two potential but not mutually exclusive causes, changes in soil chemistry caused by the invasive species, and reductions and shifts in the mycorrhizal community due to reduced native plant hosts. For example, tamarisk is known to increase soil salinity (Meinhardt &

Gehring 2012), and *Solidago canadensis* (Canada goldenrod) can shift the fungal community to one that promotes its own competitiveness over that of native plants (Zhang et al. 2010). Due to the variety of benefits associated with native mycorrhizal symbioses, reductions and shifts in their communities due to invasive species and other disturbances are concerning, particularly as the stressors on plant communities increase under climate change. For example, mycorrhizal fungi have been implicated in increasing plant access to nutrients; mediating responses to stressors, pests, and climate (Babikova et al. 2013; Kivlin et al. 2013; Wilkinson and Dickinson 1995); providing access to deep water (Querejeta et al. 2007); moving water among plants to where it is needed (Egerton-Warburton et al. 2007); improving plant water use efficiency (Querejeta et al. 2003; 2006); sharing carbon resources among plant species (Klein et al. 2016, and altering interspecific tree dynamics (Teste & Simard 2008). However, we could find no studies that identify whether the eradication of invasive species permits recovery of the mycorrhizal communities and their services or how long natural regeneration of these communities might take after the invasion by an exotic plant species has been remedied.

We found a few studies suggesting that mycorrhizal fungal communities on planting material are different from or less diverse than those growing in natural populations (Sykorova et al. 2007; Southworth et al. 2009), and that dispersal challenges may preclude meaningful recruitment and recovery of natural mycorrhizal communities, even after five years (Peay et al. 2010; Pankova et al. 2018). None of these studies specifically address the changes caused by invasive species, but they do suggest reasons why natural recovery of mycorrhizal communities after ecosystem invasion by exotic plant species could take an extended period of time. Since the outcomes of symbioses change with every change in symbiotic partner and environmental condition (Rillig & Mummey 2006), such constraints on the natural recovery of mycorrhizal

fungal communities could represent crucial deficiencies in habitat restoration and regeneration that account for the benefits of active restoration of mycorrhizal fungi.

Actively restoring diverse mixes of appropriate native mycorrhizal fungal communities is suggested by multiple studies and meta-analyses to promote survival, growth, and diversity of plant communities if thoughtfully incorporated into restoration planning in conjunction with plant provenances, palettes and site conditions (e.g. Wubs et al. 2016; Rua et al. 2016; Koziol and Bever 2017). At least one meta-analysis has shown that the sizable benefits seen also improve with time since planting (Neuenkamp et al. 2019). These findings concur with broader science reflecting that the ability of organisms to survive and adapt is likely dependent on the confluence of their own traits and adaptations with those of their microbiome (Zilber-Rosenberg & Rosenberg 2008; Bordenstein and Thelis 2015; Whitham et al. 2020). In fact, because of their contributions, shorter life-cycles, and prolific genetic sharing, microbiota like mycorrhizal fungi may be particularly crucial to promoting swift adaptation to change and rehabilitation of degraded systems (Wilkinson & Dickinson 1995; Coban et al. 2022).

However, consideration of factors including timing, soil, plant species and plant provenance, and other site characteristics have proven integral in achieving optimal results when implementing mycorrhizal restoration (e.g. Johnson et al. 1992; Maltz and Treseder 2015; Mortimer et al. 2005). Neutral to negative effects occur when these factors are not adequately addressed, when plant roots fail to gain direct contact with live mycorrhizal fungal roots, or mass-produced products are utilized (e.g. Rua et al. 2016, Maltz and Treseder 2015, Saloman et al. 2022). Thus, it remains unclear how well optimized plant and fungal pairings must be, and how assisted migration and the tendency for co-adaptation between local soil, mycorrhizal fungi, and plants indicated by other studies (e.g. Johnson et al. 1992; 2010; 2014; Rua et al. 2016) will interact. Filling these knowledge gaps can inform the cost/benefit calculations for implementing

active restoration of native mycorrhizal communities when it is needed (for example, see Hart et al. 2017 Figure 4), and assist in the design of inoculum and plant provenance combinations that best harness biotic co-adaptation and site conditions.

To address these knowledge gaps, we investigated the interactions between a confluence of experimental treatments including: assisted migration, mycorrhizal inoculation, and soil legacies left by invasive tamarisk under stressful drought conditions. We focus here on Fremont cottonwoods due to their foundational nature in riparian communities of the arid southwest, extensive data on their ecotypes and ecophysiology, and the immense loss of riparian areas experienced in the U.S. Riparian arteries of the southwestern U.S. comprise less than 0.5% and 2% of the land area in Arizona and the southwest, respectively, but support a disproportionate 60 to 75% of the wildlife (Arizona Riparian Council 2004, Poff et al. 2012). Best estimates placed the loss of these riparian habitats at 90% (Zaimes 2007). Previous studies have also demonstrated that warmer, sub-optimal temperatures reduce canopy gas exchange via stomatal conductance (Grady et al. 2013), increase plant stress (Hultine et al. 2013), and can generally be expected to create predictable, linear losses in tree productivity (Grady et al. 2011) in these habitats.

We used provenance trials with assisted migration to replant a riparian corridor and floodplain after tamarisk invasion. We planted trees from six populations or provenances, three each from two contrasting Freemont cottonwood ecotypes (warmer SD and cooler MR) at a cooler MR ecotype site. Given prior research regarding the negative impacts of tamarisk neighbors on native cottonwoods, concurrent with the ability of tamarisk to reduce and shift mycorrhizal fungal communities and change soil chemistry (Meinhardt & Gehring 2012), we hypothesized that 1) the soil legacy left by prior tamarisk invasion would similarly reduce plant survival, even after tamarisk removal. Based on prior research demonstrating the disruption of mycorrhizal symbioses by invasive species including tamarisk (Meinhardt & Gehring 2012) and

benefits of mycorrhizal restoration (Wubs et al. 2016; Neuenkamp et al. 2019), we hypothesized

that 2) mycorrhizal restoration would improve the survival of trees in areas with a tamarisk soil

legacy. Since even the areas of the site without prior tamarisk presence had been used as a gravel

pit, contained other invasive species such as *Alhagi maurorum* (camelthorn), and contained no

remaining, live cottonwoods to provide immediate access to appropriate mycorrhizal fungal

inoculum, we hypothesized that 3) inoculation with a diverse mix of living mycorrhizal fungi

native to riparian areas in the state would also benefit trees in areas without a tamarisk soil

legacy, regardless of tree ecotype and provenance. Given prior research showing that assisted

migrant cottonwoods show 30% lower survival, we hypothesized that 4) assisted migrant trees

from the warmer SD ecotype would always have lower survival rates than trees from the local

MR ecotype, regardless of tamarisk and inoculation treatments.

Methods

Common garden planting site and source materials

The Black Rock Flow Stops common garden planting site (35.71923, -111.3194)

comprises 22 Ha in a riparian corridor and flood plain near the Little Colorado River near

Cameron, AZ. The site is falls within the cooler Mogollon Rim ecoregion (MR) of Freemont

cottonwoods (*Populus fremontii)*, and the portion of the site without tamarisk trees had been

used as a gravel pit since at least 2007. Cottonwood cuttings were collected from trees at least 20

meters apart during the 2014-2015 winter.

Cuttings were collected from six populations across the two ecoregions (Mogollon Rim,

MR; Sonoran Desert, SD) of *Populus fremontii* within Arizona (Blasini et al. 2021; 2022). The

soil and climate conditions at the common garden and tree source populations are shown in

Table 3.1. The three source populations from the SD ecoregion have mean annual temperatures

(MAT) that are warmer than the common garden planting site (Table 1; ranging from 0.1° to

1.8°C warmer, as measured in 2018). The three source populations from the MR ecoregion have mean annual temperatures (MAT) that are the same as, or cooler than, the common garden planting site (Table 1; ranging from 0° difference to 5.1°C cooler, as measured in 2018). Cottonwoods from the cooler, Mogollon Rim (MR) ecotype and adaptive trait syndrome are known to have more conservative hydraulic strategies associated with frost protection (and potentially risk reduction under drought but not warming (Blasini et al. 2021; 2022). Cottonwoods from the warmer, Sonoran Desert (SD) ecotype and adaptive trait syndrome are known to have traits that maximize hydraulic efficiency and evaporative cooling, which may pose challenges under droughts of increasing frequency or severity (Ikeda et al. 2017, Blasini et al. 2021; 2022).

Cuttings were propagated and grown in the greenhouse for two years in 10.16 cm by 76.20 cm pots to encourage deep root systems. Tamarisk was removed from the tamarisk areas of the site with bulldozers during the spring and summer of 2017. Planting holes were dug 152.4 cm deep to plant cottonwoods as close to the water table as possible and preclude the need for irrigation. Trees were planted during November and December 2017. Replicates of trees stratified across ecotype and populations were planted in random order within blocks, and replicated in five blocks for each treatment. Trees were all approximately 150 cm tall (plus 76 cm of roots and potting soil) at the time of planting. Paired inoculated and uninoculated blocks within the tamarisk and non-tamarisk areas were separated by a minimum of 10 m to maintain inoculation treatment integrity over the two-year experiment. Cottonwood trees were planted with or without live mycorrhizal soil inoculum (sourced from the Bullpen population within the SD ecoregion and bulked-up in the greenhouse) due to its lack of history with tamarisk and healthy cottonwood population. In conjunction with the deep planting methodology, trees were watered when planted but received little water after planting aside from natural precipitation. In

addition to arid climate of the region, and lack of irrigation provided to the trees in the experiment, at the time of the experiment the southwestern United States, where the common garden is located, experienced the worst drought since 800 ce (Williams et al. 2022).

Locations of the common garden, and inoculum and tree source populations, are shown in Figure 3.1. Climate and soil data for the common garden and all source populations is presented in Table 3.1. The source ecoregions of trees (Sonoran Desert (SD) in shades of red, and Mogollon Rim (MR) in shades of blue), and the difference in mean annual temperature (MAT) between the natural populations from which trees were sourced and where they were planted (MAT transfer distance) is utilized in graphs to depict the change in climate that trees experienced (similar to the use of DD5 in Cooper et al. 2019 and MAT in Blasini et al. 2022, for easy comparison).

Survival and growth measurements

Tree metrics for the black rock flow stops common garden were sampled in July 2018 (year 1) and September 2019 (year 2). Survival was recorded for each tree, and height and diameter at root collar (DRC) were measured for each shoot emerging from the ground for each tree. Calipers were used to measure DRC, and area at root collar (ARC) was calculated from DRC (ARC $= 2\pi r^2$ where r $=$ DRC/2). Volume for each tree was calculated as the sum of the volumes each shoot (as a cylinder, based on height and ARC).

Greenhouse experiment to assess inoculation efficacy

Since roots were unable to be sampled at the field site (due to planting depth and immediate downward growth of tree tap roots), we conducted a greenhouse experiment that replicated the field study as closely as possible to confirm the efficacy of mycorrhizal inoculation utilizing the same methods and soils. In the greenhouse experiment, 80 trees (10 in each tamarisk and inoculation combination) were planted in 50% potting soil and 50% soil from the black rock

flow stops common garden from either the tamarisk or no tamarisk areas. Live or sterilized

mycorrhizal soil inoculum from the SD ecoregion (sourced from Agua Fria and bulked-up in the

greenhouse) was added to each potting combination. Trees in each greenhouse treatment were

stratified across the same ecoregions (and populations whenever possible) as in the field

experiment. Trees that died during the course of the 125 day experiment were replanted to

maintain a balanced design.

Efficacy of inoculation based on greenhouse trees

To confirm the efficacy of the inoculation methods used in the field, fine roots from

living greenhouse trees were gathered 125 days after initiating treatments. Roots were placed

into plastic bags, and immediately frozen. Roots from all samples were washed in a mixture of

0.01% Tween (Fisher Scientific, Inc.) and RO water, and then RO water and to remove debris.

Roots samples were frozen for preservation until they could be treated and evaluated

individually. Since EMF have been shown to be more sensitive to tamarisk treatments

(Meinhardt & Gehring 2012), EMF colonization was evaluated for trees in all treatments.

EMF root tips were visually classified and counted under a dissecting microscope using

the gridline intersection method (Brundrett et al. 1996). For 75 trees, 7500 gridline intersections

were evaluated, and 258 EMF root tips were visually categorized and counted.

Statistical analyses

All statistical analyses were conducted in R version 4.0.3 (R Core Team 2020).

Individual trees were used as the independent experimental unit since plants were randomly

planted and environmental heterogeneity was relatively low (as in Grady et al. 2011).

To investigate the efficacy of inoculation, EMF colonization rates from the greenhouse

were assessed using mixed effects linear regression with the lmer() function in the lme4 package

(Bates et al. 2015) in R. Inoculation treatment was used as the predictor variable, and population was used as a random variable.

In exploratory data analyses multiple covariates including genotype, garden block, soil moisture, prior tamarisk experience of source populations, the perennial versus ephemeral nature of water sources in the tree source populations, failed to change the patterns seen in the results or meaningfully improve the statistical evaluation of treatment results. Evaluating the full three sets of multiple pairwise comparisons embedded in the experimental design was logistically prohibitive and not required to answer the hypotheses. Thus, statistical analyses were limited to addressing the hypotheses and factors which the study was designed to evaluate. During initial data exploration, it became apparent that the survival of inoculated assisted migrants during year two might be the same as or higher than that of trees from the local ecoregion, so this specific comparison was addressed for the last hypothesis. Due to extremely high mortality reducing available sample sizes for growth metrics, statistical analyses focus on survival, where the most revealing results are found.

To analyze binomial survival data by treatment variables in conjunction with the random effects of tree source population, binomial regression was used. Individual binomial regressions were assessed using the glmer() function in the lme4 package (Bates et al. 2015) in R. For each test, tree source population was used as a random variable, and the data was filtered and individual predictor variable selected specific to the relevant hypothesis. Measures of effect size were obtained utilizing the odds ratios from the emmeans() function in the emmeans package (Length 2020), specifying type='response' to obtain results that were back-transformed from the logit scale.

Results

Analysis details and results are presented in Table 3.2. Significant results are bolded.

Effectiveness of inoculation treatment

EMF colonization rates were significantly higher in inoculation treatments than in treatments without inoculation (F = 18.52, df = 1, 73, and p < 0.001). Inoculation accounted for approximately 20% of the variation in the proportion of EMF root tips (adjusted $R^2 = 0.19$).

H1: Tamarisk soil legacy negatively impacts cottonwood survival

Without inoculation treatments, trees in tamarisk were significantly less likely to survive year one (Z = -&.623, p < 0.0001). As depicted in Figure 3.2, survival of trees in tamarisk soil was 14% of that for trees in soil without a tamarisk legacy.

H2a: Inoculation increases survival in tamarisk legacy soil

For trees in tamarisk soil, year one survival was significantly higher with inoculation than without (Z = -2.718, and p < 0.01), as was year two survival (Z = -2.177, and p < 0.05). As shown in Figure 3.3, survival in tamarisk soil if inoculated was 208% and 361% of that for trees that were not inoculated during years one and two, respectively. However, the benefits of inoculation are clearly higher in trees from the same ecotype as the inoculum (SD), as reflected in Figure 3.3. and significant effect of inoculation (Z = -3.507, p < 0.001) and the interaction between inoculation and tree ecotype (Z = 2.206, p < 0.05).

H2b: Inoculation increases survival in soil without a tamarisk legacy

For trees in soil without a tamarisk legacy, year one survival was significantly lower with inoculation than without (Z = 3.254, and p < 0.01). As shown in Figure 3.4, year one survival in soil without a tamarisk legacy if inoculated was 51% of that for trees that were not inoculated.

For trees in soil without a tamarisk legacy, year two survival was not significantly different overall for inoculated and un-inoculated trees (Z = 0.435, and p < 0.67). However, Figure 3.4 depicts visibly positive responses to inoculation for SD trees and neutral to negative responses for MR trees, reflecting a main effect of inoculation (Z = -2.662, p < 0.01) and

interaction between inoculation treatment and tree ecotype (Z = 2.206, p < 0.05) when both inoculation and tree ecotype are included in the model with a random effect of population.

H3: Assisted migrants always have lower survival than trees from the local ecoregion

During year two, survival for inoculated trees was significantly higher in assisted migrant populations, regardless of tamarisk soil legacy status (Z = 3.14, and p < 0.01). As shown in Figure 3.5, survival of inoculated trees during year two for assisted migrants was twice that for trees from the local ecoregion.

Discussion

In this study, the soil legacy left by tamarisk invasion reduced survival of native cottonwood trees by 85%, even after tamarisk trees were removed from the area. In areas with this tamarisk soil legacy, inoculation with a diverse mix of native mycorrhizal fungi appropriate to riparian areas doubled and then tripled cottonwood survival, averaged across trees from both ecoregions and all populations. In non-tamarisk legacy soils, the effects of inoculation overall was not significant, visibly varying and including neutral to negative results, interacting with timing and the pairing between tree source and inoculum source, emphasizing the importance of refining the understanding of optimal pairings between site conditions, plant source, and fungal inoculum source. During the second growing season, assisted migrant trees inoculated with an appropriate, diverse mix of native mycorrhizal fungi from their home ecoregion survived extremely harsh drought conditions in the field at twice the rate of local trees, demonstrating that appropriate mycorrhizal inoculation could increase the efficacy of assisted migration as a climate change adaptation strategy.

The effects of invasive vegetation

Our study provides evidence that soil legacies left by an invasive species such as tamarisk can impact native plants negatively long after the removal of the invasive species itself. This is

consistent with the literature showing that invasive species reduce and shift mycorrhizal communities, reduce plant survival and/or biomass, and impact mycorrhizal activity (Carey et al. 2004; Meinhardt & Gehring 2012; Wilson et al. 2012), the success of post-disturbance replanting in conjunction with restoration of the mycorrhizal community (Wubs et al. 2016; Koziol and Bever 2017), and evidence that the effects of at least some disturbances on mycorrhizal communities can last for many years (Pankova et al. 2018). While the negative effects of many invasive species on native plants and on mycorrhizal mutualisms has been documented across multiple systems, further research is needed to confirm that the soil legacies of these same invasive plants continue to harm native plants after the removal of the plants themselves in all of these same ecosystems.

Implications for assisted migration

Though it is challenging to address multiple stressors at once within field studies, studies like the current one are crucially important to understanding what results can be expected from many interacting factors. In this study, we have shown that small, incremental steps in assisted migration (Grady et al. 2015), even across ecotype or adaptive trait boundaries (Cooper et al. 2019; Blasini et al. 2021), can be similarly successful to local plant provenances under multiple stressors, if given appropriate mycorrhizal inoculum. This is consistent with results from Remke et al. (2020) suggesting that inoculation with microbiota from a plant provenance's home environment, while most effective in home soil, can help ameliorate the negative impacts of growing at novel hotter, drier sites for ponderosa pines (*Pinus ponderosa*). This reinforces the merits of considering the confluence of the organism and its microbiome in replanting, restoration, and climate change adaptation (Zilber-Rosenberg & Rosenberg 2008; Bordenstein and Thelis 2015; Whitham et al. 2020), and the urgent need for large-scale assisted migration studies that address inter-species interactions (Bucharova 2017). For example, the results of the

current experiment indicate that increasing genetic diversity by using some amount of assisted migration with appropriate mycorrhizal inoculation in restoration is a promising strategy for climate change adaptation. However, more research is needed to determine the long-term effects of assisted migration on the ability of trees to form common mycorrhizal networks that exchange nutrients, water, and pest signals (e.g. Bingham & Simard 2011; Babikova et al. 2013; Klein et al. 2016), and how that is affected by including plants and mycorrhizal fungi from multiple provenances, for example.

The impact of time and timing

This study found clear evidence of the effects of time and timing on the benefits of mycorrhizal restoration. We are aware of at least two studies demonstrating that the timing of inoculation can impact its initial effectiveness, due to the fact that establishing the mycorrhizal symbiosis, particularly under otherwise stressful conditions, imposes an initial cost on the plant (Mortimer et al. 2005; Maltz & Treseder 2015). However, the clear impacts of timing on field survival for trees in non-tamarisk soil in the current study (negative the first growing season, and visually varying during the second growing season by provenance for no significant effect overall) demonstrate the importance of this consideration in a manner that we have not seen in the literature to date. Further research is required to identify the optimal timing and methods of mycorrhizal inoculation during the restoration timeline. Similarly, the finding that the overall beneficial effects of inoculation for trees in tamarisk legacy soil grew with time (doubling survival in the first season and tripling it during the second growing season) concur with findings from Neuenkamp et al. (2019) that the beneficial effects of needed and appropriate inoculation can grow with time.

Interactions between site conditions, and provenances of native plants and mycorrhizal fungi

Given extensive evidence showing that mycorrhizal symbioses are impacted by and co-evolved with plant provenances and site conditions (Johnson et al. 1992; Johnson et al. 2010, Johnson et al. 2014; Rua et al. 2016), it is arguably unsurprising that in non-tamarisk soil the effects of mycorrhizal inoculation depended on the pairings between the provenance of plants and the mycorrhizal fungi utilized. However, due to the widespread success of mycorrhizal restoration (Neuenkamp et al. 2019), the presence of other invasive plant species and the lack of remaining cottonwood trees to provide fungal inoculum at the site, and the continued prevalence of mass-produced mycorrhizal inoculums (Hart et al. 2017; Saloman et al. 2022), it seemed reasonable to hypothesize that inoculation with a diverse mix of native mycorrhizal fungi native to cottonwood trees in a riparian area in the same state would be broadly beneficial across plant provenances. Interestingly, even taking into consideration the effects of timing, our study did not support this. Further research is needed to reveal how pairing mycorrhizal inoculum sources and plant provenances can optimize results. For example, developing inoculum combinations that include microbiota from high salinity sites could be used to assist paired plant partners at a replanting site with high salinity, but it is not yet apparent whether plants of all intraspecies provenances would benefit equally, or what combination of mycorrhizal inoculation sources would best promote plant provenances of varying sources. As best practices for restoration begin to incorporate mycorrhizal restoration, there is an urgent need for additional research on mismatched verses optimal inoculum-plant pairings to avoid unintended, counterproductive negative impacts from a tool that has so much potential. Since the outcomes of mycorrhizal symbiosis are known to vary with every change in partner and environment (Rillig & Mummey 2006), specific, limited subsets of mycorrhizal fungi within a community are known to provide certain services (e.g. Egerton-Warburton et al. 2007); and physiological strategies of plants can vary by ecotype and population (e.g. Blasini et al. 2021; 2022), further attention might be

required to optimally pair the physiological adaptations of plant provenances and populations with the strategies and services provided by fungal members of different provenances.

Conclusions

This study demonstrated a large afterlife effect of an invasive species on habitat restoration, and that field survival of assisted migrant plant provenances can be boosted beyond that of local plant provenances by implementing intentional assisted migration of their mycorrhizal fungi. These findings improve our understanding of fundamental ecological concepts about how invasive species and mycorrhizal symbioses affect ecosystems, and provide restoration best practice targets. The interactions seen among timing, site conditions, and plant and mycorrhizal fungal community provenances suggest multiple lines of future research to optimize the practice of active restoration of native mycorrhizal communities, and further inform knowledge of fundamental principles underlying the coordination of plant physiological adaptations and traits with the services provided by their mycorrhizal fungi.

Acknowledgements

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Tables and figures

Table 3.1: Climate and soil characteristics for the common garden and source populations.

Source Population, River (Code)	Keams Canyon, Little Colorado River (KKH-OPI)	Jack Rabbit, Little Colorado River (JLA-JAK)	Citadel Wash, Little Colorado River (CLF-LCR)	Black Rock Flow Stops Common Garden, Little Colorado River (LCR)	Kingman, Willow Creek (KWF-WIL)	Bullpen, Clear Creek (BCE-BUL)	Horseshoe Ranch, Agua Fria (CAF-AUG)
Ecotype / Adaptive Trait Syndrome	MR	MR	MR	MR	SD	SD	SD
Latitude	35.81152	34.96	35.6088	35.71923	35.143	34.53972	34.25671
Longitude	-110.16958	-110.436	−111.31369	-111.3194	-113.54284	-111.6966	-112.06617
Elevation (m)	1982	1510	1349	1331	1210	1254	1061
Precipitation (mm)	160.07	165.03	174.86	139.54	180.98	364.11	364.68
Minimum Temperature (°C)	2.7	4.8	8.5	8.6	7.6	9.2	10.9
Maximum Temperature (°C)	19.8	23.6	24.1	24.1	25.2	24.9	25.4
MAT (°C)	11.2	14.2	16.3	16.3	16.4	17.1	18.1
MAT Transfer (°C)	5.1	2.1	0	-	-0.1	-0.8	-1.8
Max VPD (hPa)	21.39	27.73	28.98	28.7	29.52	28.9	29.12
Soil Series	Tewa very fine sandy loam	Ives Soils	Jocity-Joraibi-Navajo-Riverwash complex	Jocity-Joraibi-Navajo-Riverwash complex / Torrifluvents, saline	Arizo-Franconia-Riverwash complex	Anthony fine sandy loam	Barkerville Cobbly Sandy Loam
Soil Content	17.5% clay, 59.3% sand, 23.2% silt	71% sand, 17% silt, 13% clay	35% clay, 40% sand, 25% silt	35% clay, 40% sand, 25% silt	11.5% clay, 65.7% sand, 22.8% silt	2.8% clay, 67.5% sand, 21% silt	68% sand, 20% silt, 13% clay
pH	7.6	8.2	8.2	8.2	7.9	8.2	7
Electrical Conductivity (dS / m)	1	20	0.7	0.7	1	1	0

Notes

1. Climate data is for 2018 is from PRISM Climate Group (2020).
2. Soil data is from United States Department of Agriculture (USDA), Natural Resources Conservation Service (2020).
3. Climate abbreviations are as follows: mean annual temperature (MAT) vapor pressure deficit (VPD).
4. Ecotype/adaptive trait syndrome is as described in Blasini et al. (2021; 2022).
5. Soil content, pH and salinity were not available for the exact land parcel comprising the common garden (Torrifluvents, saline). Thus, characteristics of the immediately adjacent soil series appear here. Salinity measurements from within the common garden itself can be found in the results section.

Table 3.2: Analysis details and results for inoculation efficacy, and each hypothesis.

Hypothesis	Filters	Predictor Variable	Test	Year	n	Model Results	Details
EMF inoculation efficacy	-	Inoculation treatment	lm()	-	75	F = 18.52, df = 1, 73, and p < 0.001	Inoculation accounted for 20% of the variation in rates of EMF colonization, averaged over tamarisk treatments.
H1: Tamarisk has negative impacts on cottonwood survival.	Trees without inoculation	Tamarisk treatment	glmer()	1	471	Z = -7.62, p <0.0001	Survival of trees in tamarisk soil is 14% of that for trees in soil without a tamarisk legacy.
H2a: Inoculation incrases survival in tamarisk legacy soil.	Trees in tamarisk legacy soil	Inoculation treatment	glmer()	1	474	Z = -2.72, p<0.01	Survival in tamarisk soil if inoculated is 208% of that if not inoculated.
				2	73	Z = -2.18, p<0.05	Survival in tamarisk soil if inoculated is 361% of that if not inoculated. However, benefits of inoculation are clearly higher if trees are from the same ecotype as the inoculum (SD), as depicted in Figure 3.3 and reflected in the significant effects of inoculation (Z = -3.51, p < 0.001) and the inoculation by source interaction (Z = 2.21, p < 0.01) if tree ecotype is included in the analysis.
H2b: Inoculation increases survival in soil without tamarisk legacy.	Trees in soil without a tamarisk legacy	Inoculation treatment	glmer()	1	470	Z = 3.25, p<0.01	Survival in non-tamarisk soil if inoculated is 51% of that if not inoculated.
				2	246	Z = 0.44, p = 0.67	No significant effect overall. However, including tree ecotype in the analysis yields a significant effect for inoculation (Z = -2.662, p < 0.01) and the interaction between ecotype and inoculation (Z = 2.206, p < 0.05), as can be seen in the visually positive effect of an SD inoculant on SD trees, and the neutral to negative effects of the SD inoculum on MR trees in Figure 3.4.
H3: Assisted migrants always have lower survival than trees from the local ecoregion.	Inoculated trees that survived the first field season	Ecoregion source	glmer()	2	117	Z = 3.14, p < 0.01	Survival of assisted migrants was twice that of those from the local ecoregion.

Figure 3.1: Map of cooler, Mogollon Rim (MR; blue) and warmer, Sonoran Desert (SD; red) ecotype source locations and black rock flow stops common garden (in the cooler, MR ecoregion). Inoculum source is indicated with (I).
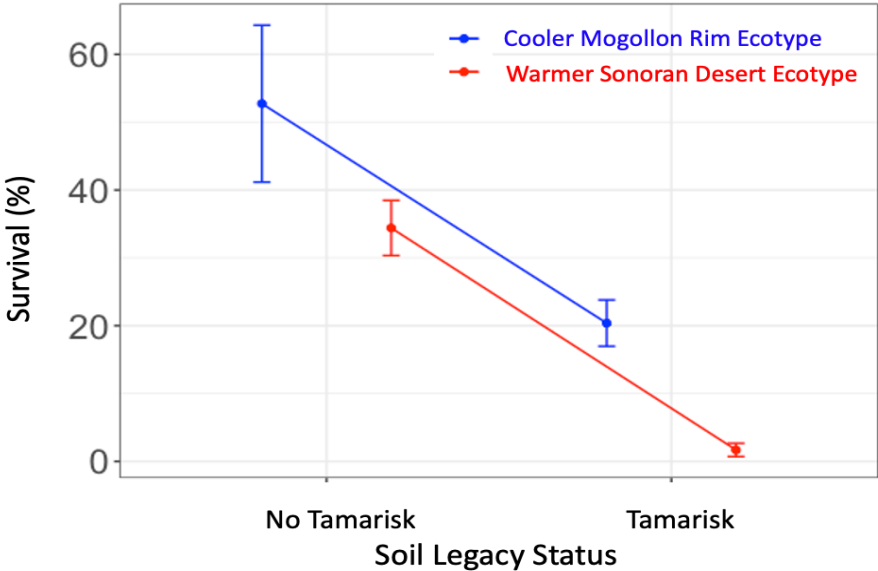

Figure 3.2: First year survival for trees in the common garden sourced from the cooler, local Mogollon Rim (MR; blue) ecoregion and from the warmer Sonoran Desert (SD; red) ecoregion in each tamarisk treatment (without mycorrhizal inoculation). Dots are treatment/source means and error bars represent 1 SE. Across ecoregions and source populations, first year survival for trees in tamarisk soil was 14% of that for trees in soil with no tamarisk legacy (p<0.001).
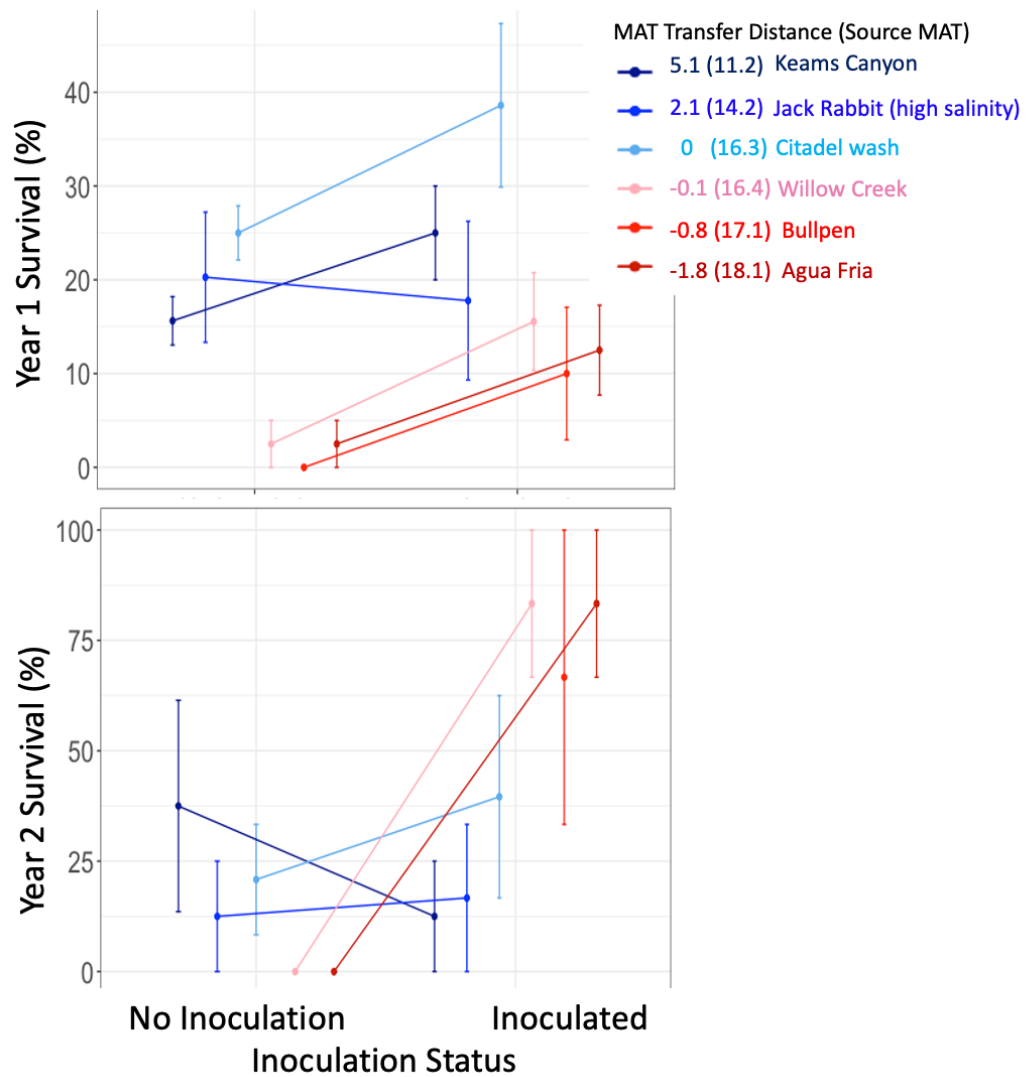
Figure 3.3: First and second year survival (top & bottom, respectively) for trees in the common garden tamarisk soil blocks. Shades of blue show results for trees sourced from populations in the cooler Mogollon Rim (MR) local ecoregion and shades of red show results for assisted migrant trees sourced from the warmer Sonoran Desert (SD) ecoregion. Dots are population/inoculation treatment means and error bars represent 1 SE. Inoculation doubled year one survival in tamarisk soil ($p<0.01$). Inoculation triples year two survival in tamarisk soil ($p<0.05$).
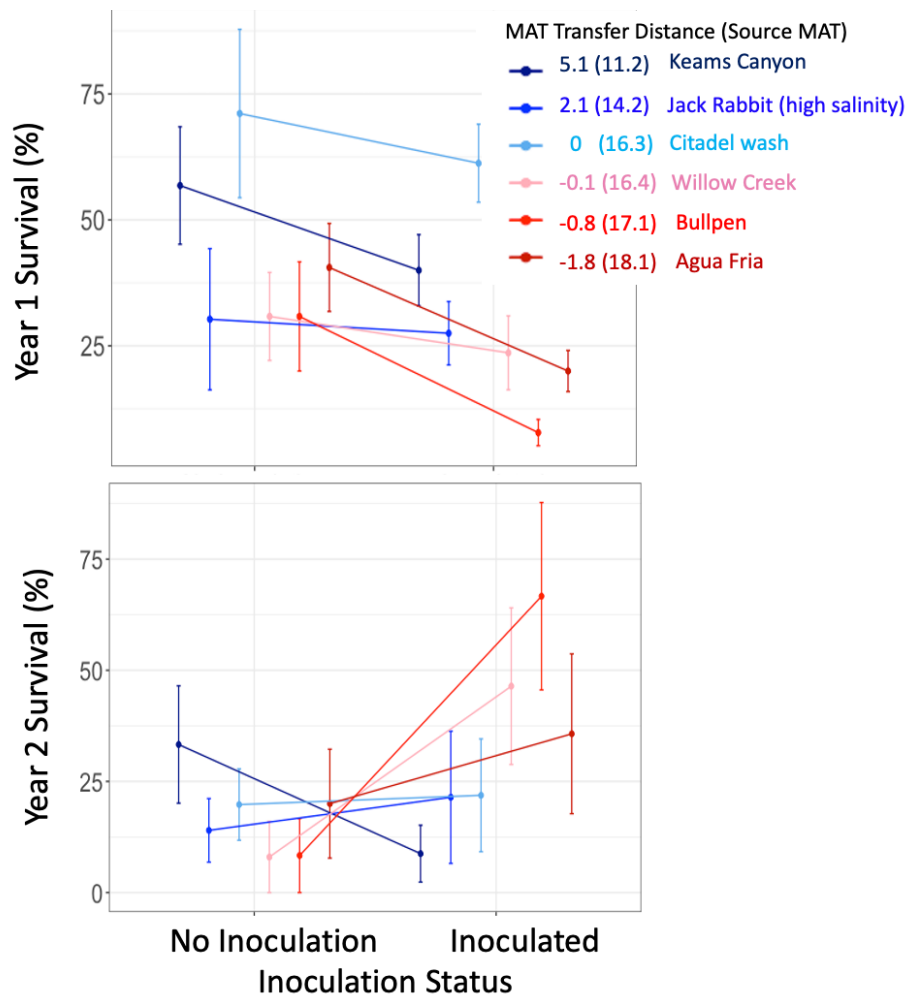.

Figure 3.4: First and second year survival (top & bottom, respectively) for trees in the common garden blocks without a tamarisk legacy. Shades of blue show results for trees sourced from populations in the cooler Mogollon Rim (MR) local ecoregion and shades of red show results for assisted trees sourced from the warmer Sonoran Desert (SD) ecoregion. Dots are population/inoculation treatment means and error bars represent 1 SE. During the first year, inoculation reduced survival by one half (p<0.01), and during the second year inoculation had no significant effect overall but results reflect visually differing slopes between ecoregions, reflecting an interaction between inoculation and tree ecotype (p<0.05).
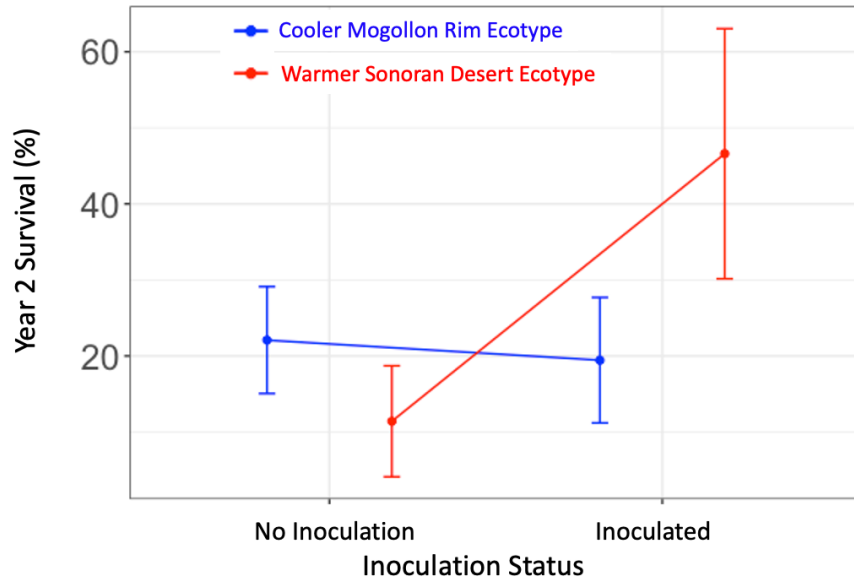
Figure 3.5: Second year survival for trees in the common garden sourced from the cooler, local Mogollon Rim (MR; blue) ecoregion and from the warmer Sonoran Desert (SD; red) ecoregion in each inoculation treatment. Dots are treatment/source means and error bars represent 1 SE. Assisted migrant trees from warmer populations (SD) had a higher chance of surviving during the second year than trees from the cooler, local ecoregion (MR) if inoculated with an SD inoculum, regardless of tamarisk soil treatment ($p<0.01$).