Mutualist matters: mycorrhizal influences on water stress in oak seedlings

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Introduction

Mycorrhizal associations are ancient symbioses between plants and fungi. The first clear fossilized evidence of mycorrhized rhizomes dates to 407 million years ago in the Devonian period, however there is some indication these relationships originated earlier, perhaps even arriving with plants to land (Brundrett and Tedersoo 2018). The transaction generally is one of mutualistic reciprocity: plants provide photosynthetic sugars to fungi and fungi relay soil resources to plants (Smith and Read 2007). Soil resources commonly include low-mobility nutrients such as phosphorus, which some plants receive entirely from their mycobiont (Santander et al, 2017). Under drought conditions mycorrhizae often ameliorate water stress (Augé 2001; Jayne and Quigley 2014; Lehto and Zwaizek, 2011).

Most plants associate with one general type of mycorrhizal fungi. The fungi of the evolutionarily more primitive lineage of arbuscular mycorrhizae (AM) are obligate symbionts, which form specialized structures within the root cortical cells of more than 70% of all vascular plant species (Brundrett and Tedersoo 2018). By contrast, the more diverse group of ectomycorrhizal fungi (EM), which evolved independently at least 60 times (Raven and Andrews 2010), remain external to the root cells and form hyphal mantels around plant root tips. The prominent morphological and physiological differences between AM and EM confer complementary benefits to the plant, for instance, AM likely require less carbon to maintain (Leake et al 2004) (they do not produce fruiting bodies), scavenge inorganic nutrients (DeForest and Snell 2020), and are more resilient to higher temperatures and extremes in soil moisture (Teste et al, 2019) whereas many EM mine nutrients directly from soil organic matter (Read and Pérez-Moreno 2003) and may offer more protection from pathogens by physically enveloping root tips (Marx 1972). Though mycorrhizal relationships are thought to be highly phylogenetically conserved within plant families or genera, some taxa of plants are capable of dual mycorrhization (Teste et al. 2019), including the globally widespread genus *Quercus*.

Mycorrhizal fungi can mitigate plant water stress or its negative effects through a variety of mechanisms including direct uptake and transfer of soil water (Püschel et al. 2020; Marulanda et al., 2003; Egerton-Warburton et al., 2003), regulation of the phytohormone ABA (Ouledali et al., 2019) and aquaporin expression (Bárzana et al., 2014; He et al., 2015), protection from oxidative damage (Lehto and Zwaizek 2011; Santander et al., 2017), enhanced nutrition (Augé 2001; Li et al., 2021) and photosynthetic efficiency (Porcel and Ruiz-Lozano 2004; Augé et al., 2016), and improved soil physical properties (Bitterlich et al., 2018; Augé 2004). Although some studies find alleviation of drought stress through EM association, including in Fagaceae members *Castanea* (Aryal et al 2020) and *Quercus acutissima* (Li et al, 2021), it is probable that EM colonization and diversity will decrease during drought (Kilpeläinen 2017) and mycorrhizal community composition will shift towards AM predominance (Gehring et al 2006). In dual mycorrhizal *Quercus agrifolia*, rainfall variability year to year in xeric sites greatly influenced

EM colonization in the upper rhizosphere and roots became associated almost exclusively with AM during severe drought (Querejeta et al 2009).

The benefits of symbiotic association may be particularly important to germinants in water-limited environments, where fungal partners can help seedlings survive the most vulnerable first weeks of life (Pereira et al., 2021; Atala et al., 2012), however inocula of potential symbionts are patchily distributed and constrained by host plant availability for both AM (Whitcomb and Stutz 2007; Grünfeld et al., 2022) and EM (Pickles et al., 2012; Peay et al 2010). The proximity, identity, and age of compatible tree hosts strongly influences ectomycorrhizal community composition (Janowski et al 2022); the higher proportion of AM overstory, the more extensive the AM colonization of the understory (Grünfeld et al, 2019).

Mycorrhization promoted growth in numerous field and greenhouse studies on *Quercus*, with different outcomes depending on abiotic conditions and the taxa of available mycorrhizal fungi. Egerton-Warburton and Allen (2001) found that either AM or EM inoculum promoted *Q. agrifolia* seedlings' growth and nutrient status, but not both combined. In *Q. macrocarpa*, EM species richness and colonization decreased moving away from the forest edge, with almost no infection at a distance of 20 m (Dickie and Reich 2005) and levels of colonization correlated with increased N and growth (Dickie et al., 2005). *Q. rubra* seedlings grown beneath congeners obtained more nutrients than those grown under AM *Acer rubra* (Dickie, Koide, and Steiner 2002). Biomass of *Q. robur* seedlings correlated positively with ectomycorrhizal infection, which was not limited by phylogenetic relatedness of the tree from which growing substrate was obtained, but rather by the soil abiotic properties, however even given 20% conspecific soil, soil of AM tree provenance significantly reduced infection (Dickie et al 2006).

Mature forests contain greater phylogenetic diversity of mycorrhizal fungi (Yoo et al., 2022). Stand diversity impacts EM diversity; the greatest species richness occurs in forests with a mix of gymnosperms and angiosperms (Nguyen et al., 2016). Given the small-scale spatial variability of soil fungal communities, ecological position can substantially impact colonization rates and community structure. A greenhouse study showed priority effect occurred with heterogenous inocula sources promoting increased species richness, however homogenized inoculum resulted in competitive exclusion by less diverse, and more beneficial, mutualist taxa (Livene-Luzon et al., 2017). Some trees' fungal communities are susceptible to alteration by a heterospecific neighbor (Otsing et al 2021; Haskins and Gehring 2005; Hubert and Gehring 2008); this depends on the specific host-neighbor combinations.

In arid ecosystems, mycorrhizal inoculum of mature conspecifics may facilitate connections with the fungal mutualists most likely to promote growth and survival, as seen in *Q. liaotungensis* (Tatsumi et al., 2021). Benefit of "home" soil inoculum was also demonstrated in both greenhouse and field studies on *Quercus rugosa* and attributed to high colonization by AM fungi (Olivera-Morales et al., 2011).

Texas red oak (*Quercus buckleyi*) is endemic to juniper (*Juniperus ashei*)-oak woodlands on limestone hills from southcentral Texas into northcentral Oklahoma, with the highest remaining concentrations along the rapidly urbanizing Balcones Escarpment. Regeneration is impinged upon by overabundant white-tailed deer (*Odocoileus virginianus*), extreme drought, and oak wilt fungus (*Bretziella fagacearum*), among other threats. The seedling stage is particularly vulnerable both to herbivory (Russell and Fowler 2004) and water scarcity (Cavender-Bares and Bazzaz 2000). Proximity to mature oak and juniper trees has been shown to mitigate abiotic stress (O'Donnell et al., 2020) and juniper canopy protects seedlings from

browsing by deer (Russell and Fowler 2004), however it remains unclear what role the belowground biotic influence of oak and juniper neighbors on seedling health might be. Specifically, is the mycorrhizal community available at a germination site a significant factor in seedling growth and survival?

We do not know with which general types (AM, EM) or which specific taxonomic groups Texas red oak associate and whether these associations change with life stage or environmental variables such as soil moisture. Neither do we know whether mycorrhizal partners are shared with juniper, which is commonly regarded as an AM host, however a recent study on the effects of a closely related juniper (*Juniperus deppeana*) and *Q. rugosa* in central Mexico revealed a more than 20% overlap in both AM and EM taxa between the two trees and concluded that access to fungal networks mediated by juniper would likely enhance reforestation efforts (Bermúdez-Contreras et al, 2022).

Here I ask: Do mycorrhizal inocula affect drought response in Texas red oak seedlings? Specifically, a) does mycorrhization mitigate drought stress in oak seedlings; b) does the source of the inoculum (e.g., open herbaceous vs. woody mottes vs. closed canopy forest inoculum) influence the rate, diversity, and effect of root mycorrhization; c) does the collection site of forest inoculum (e.g., beneath conspecific oak vs. codominant juniper) influence the rate, diversity, and effect of root mycorrhization; d) does the drought treatment itself change the mycorrhization rate and diversity?

Hypotheses:

I expect that the origin of the inoculum will significantly affect seedling growth and photosynthetic rates and that the differences between inoculum treatment groups will be greater among those that are water-stressed. Seedlings inoculated with forest soil collected beneath conspecifics (O) will grow largest and have the highest photosynthetic rates under well-watered conditions, closely followed by those inoculated with forest soil collected beneath juniper (J) and those inoculated with soil from woody mottes (M); seedlings inoculated with soil from open clearings with herbaceous vegetation (C) will be slightly smaller and have the lowest photosynthetic rates. Under drought conditions, I expect seedlings with juniper inoculum to achieve a slightly larger size and exhibit the highest photosynthetic rates due to the higher proportion of compatible drought-tolerant taxa, followed by oak and woody motte-inoculated seedlings, and that clearing-inoculated will be markedly smaller with much lower photosynthetic rates.

Furthermore, I expect that percent colonization and mycorrhizal diversity will be significantly affected by both water regime and inoculum source. Mycorrhization and taxonomic diversity will be highest in the well-watered O inoculum group and be associated with the most EM proportions. Both mycorrhization and diversity will decline under water stress. These impacts will be greater with ecological distance of the inoculum from oak canopy forest, thus O > J > M > C.

Experimental Design

We investigate these questions through a two-factor semi-controlled greenhouse experiment, in which oak seedlings were treated with different inocula and then split into well-watered (W) and droughted (D) treatment groups. The four inoculation treatments were prepared from soil collected at two sites within the Balcones Canyonlands Preserve: a remnant old growth juniper-oak forest at Reicher Ranch provided inoculum from under Texas red oak (O) and juniper (J); Cortaña, which was cleared of most woody vegetation in the winter of 2003-2004, provided inocula from open areas with herbaceous plant composition (C) and woody mottes (M). Thus, there are a total of eight treatment combinations, each replicated 52 times (grand total 416 seedlings).

The physiological status of the experimental plants was assessed periodically through gas exchange measurement; growth was monitored by leaf count. After harvest, leaves were scanned to calculate leaf areas; leaves and stems were weighed for above ground biomass. Root systems are frozen pending microscopy to determine percent mycorrhized root tips and the morphotypes of the mycorrhizal fungi. DNA sequencing will be conducted on the EM morphotypes to determine their taxonomic identities.

Seed collection and preparation

Texas red oak acorns were collected throughout fall 2022 at Reicher Ranch. After discarding seeds that floated in water, each acorn was weighed, assigned a unique identification number and sown into 1.5" wide x 8.25" deep conical containers. Containers were filled with an autoclaved potting mix comprised of a blend of granite and cow manure-based compost (Geogrowers Thunderdirt), shredded coconut coir pith, and super coarse vermiculite in a ratio of 2:1:1 (*v/v/v*), respectively. Pots were watered regularly, and emergence was documented from December 2022 until March 1st, 2023.

Transplanting and Inoculation

The O and J inocula were provided by soil dug beneath elder trees (circumference at ground level ≥ 100 cm). At Cortaña, inocula were collected in open areas from the root zone of herbaceous plants at least 5 m from any woody vegetation and ≥10 m from *Quercus* (C) and from under the canopy of woody mottes within a meter of both adult *Quercus fusiformis* and juniper (M). Soil cores introduce more potential inoculant sources than other methods, including spores and other dormant life stages such as sclerotia, in addition to severed hyphal networks (Haskins and Gehring 2005). The forest inocula were collected around 30.330521, -97.936728; the herbaceous inoculum and woody inoculum came from 30.372514, -97.873492. See Maps below. For O and J inoculants 16-20 trees were selected, and soil was collected from at least two locations at the base of each tree, since aspect can affect mycorrhizal community composition (Boeraeve et al., 2019). *Quercus buckleyi* trees growing amongst juniper under mixed canopy were sampled for O; juniper trees growing at least 15 m away from the nearest *Q. buckleyi* and further than 5 m from any other oak larger than 2 cm diameter were chosen for J. Likewise for C and M inocula, 16 sites were selected each, and soil was sampled from two holes per site. Approximately 500 mL of soil including fine roots was collected at these sites. For each inoculation treatment, all samples were pooled and mixed to homogenize.

Inoculation collection occurred in two bouts, on March 31 and April 2nd; each batch was used in transplanting within one day of collection to maximize viability of mycorrhizal fungi. Between collection and transplanting the soil was kept at ambient conditions approximating field conditions and out of direct sunlight. In the week prior to transplanting, seedlings of similar size without evidence of pest damage were randomly selected and assigned to treatment groups. Initial heights were measured.

Seedlings were transplanted into 10 cm wide by 24 cm deep tree pots containing an autoclaved mixture of topsoil collected from land adjacent to Reicher, sand, and rice hulls in a 2:2:1 (*v/v/v*) ratio and 118 ml of inoculum. The substrate was previously autoclaved for 45 minutes at 121 degrees C in batches no deeper than 10 cm to ensure complete sterilization. Pots were lined at the bottom with mats of coco coir to prevent soil loss and mulched on top with rice hulls to deter fungus gnats.

The experiment was divided into blocks split by water treatment, with 16 plants (4 reps x 4 inoculant treatments) randomized in each half. There were 13 such blocks for a total replication number of N = 4*13 = 52.

Drought treatment and monitoring

Transplants were watered to saturation one day a week for seven weeks to allow them to recover from transplant stress and for infections to establish. The drought treatment was initiated at the end of May. Well-watered treatment group plants continued to be hydrated to field capacity once each week; droughted treatment group plants were not watered for the duration of the experiment.

Leaf gas exchange measurements using CIRAS-3 DC were conducted every week on a random subsample of all treatments; leaf numbers and other qualitative data were also documented over the duration of the experiment.

Harvest

Harvest began for plants undergoing drought-induced senesce in the first week of July. This rolling harvest of droughted plants continued for three weeks. On July 25th, a random subsample of 35 plants per treatment in the well-watered group were harvested. At harvest, root systems were carefully separated from the soil, placed in airtight plastic bags, and immediately stored in a freezer for later analysis of mycorrhizal infection. Leaves were counted and kept refrigerated with a wet paper towel for up to a week before scanning. All above ground tissue was dried at 70 C for at least three days and weighed.

Mycorrhization analysis

Roots were briefly soaked and carefully rinsed to remove soil particles. Fragments at least 2.5 cm long with adherent fine roots were subsampled from different parts of each plant's root system. About ten subsamples were collected for AM and about 15 for EM per plant. Microscopy analysis of mycorrhizal morphotypes' percent colonization will be conducted according to well established methods as practiced in the Gehring Lab at Northern Arizona

University. For AM this entails clearing and staining with 7.5% KOH and trypan blue. (More details here – soak, heat, etc.).

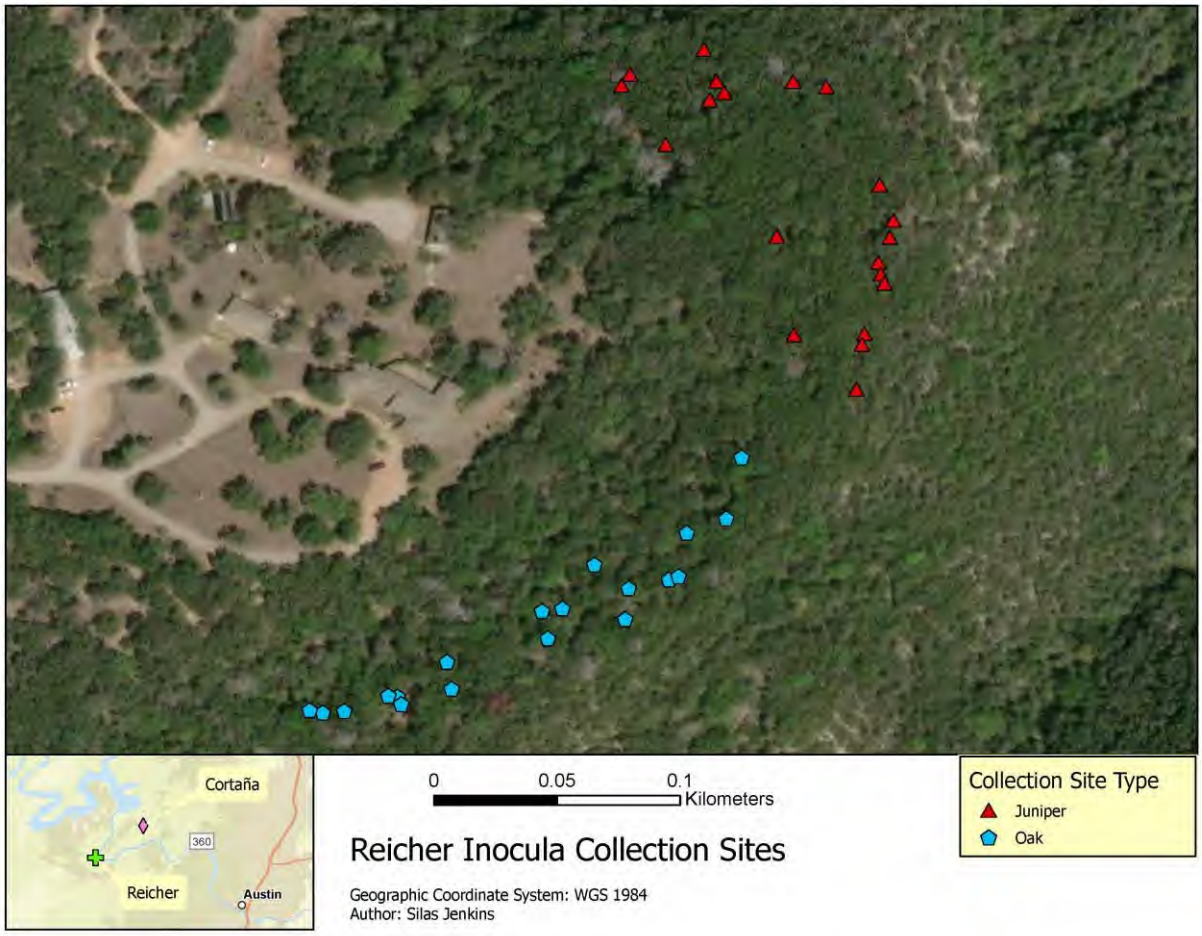
Planned statistical analysis

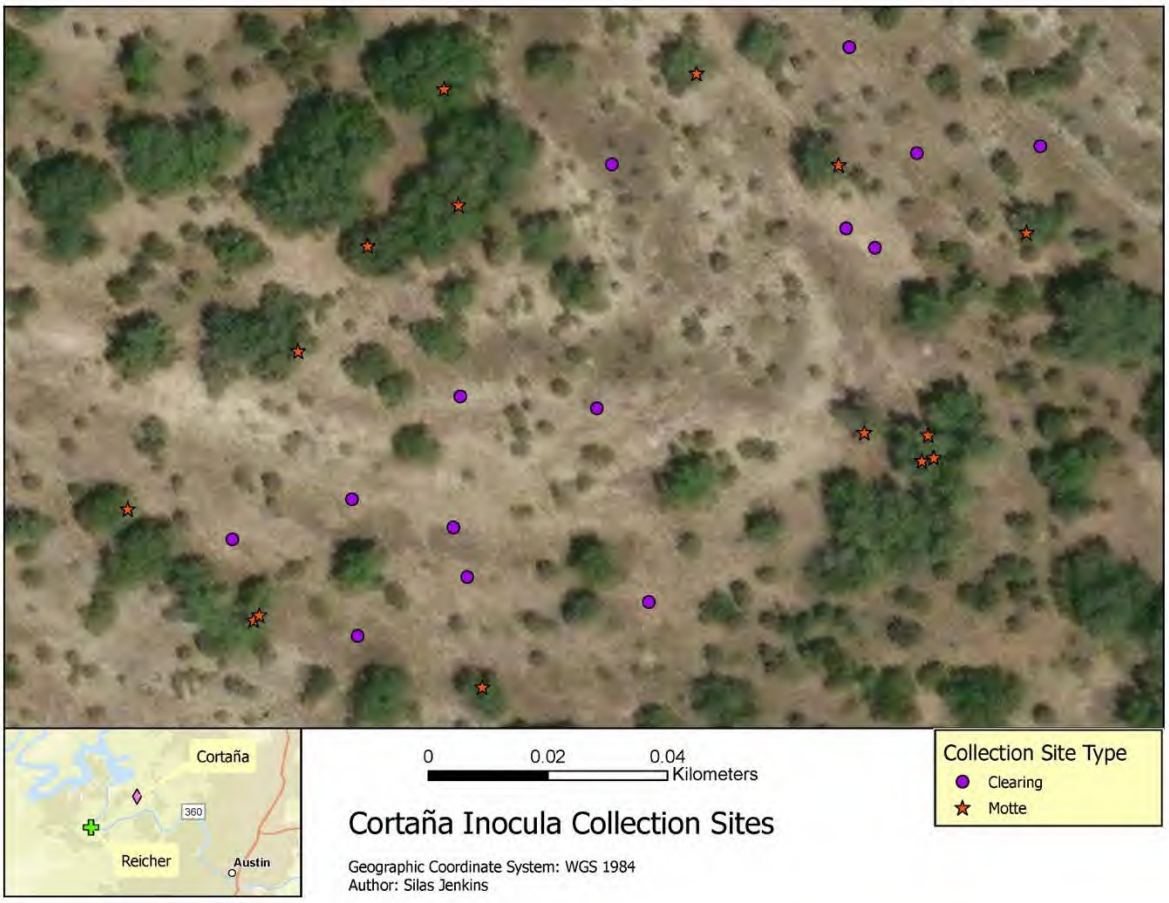
To determine treatment effects (inoculant and water) on seedling overall growth, physiological status (stomatal conductance or photosynthetic rate), I will conduct factorial ANCOVA with random effects of block. I will also test the effects of emergence time, seed weight and initial plant size to determine if model predictions significantly improve using these variables as covariates. If inoculant effects are significant, I will conduct post-hoc comparisons to determine if my hypotheses are supported. If inoculant-water interactions are significant, I will similarly evaluate my hypothesis that different inoculants produced 'most successful' seedlings in the well-watered and droughted treatments.

To answer whether the inoculum affected the rate of root colonization prior to the drought treatment, and the identity of the mycorrhizal fungi (AM vs. EM), I will use GLM to compare the likelihood of infection per root tip and the likelihood that root tips were infected with AM versus EM.

To answer whether the drought treatment affected the colonization rate and the identity of mycorrhizal fungi, I will evaluate the likelihood of colonization rates at the end of the experiment, using GLM. The overall colonization rate will be analyzed as the fraction of infected root tips per plant. Total colonization will be categorized as EM, AM or both and analyzed by Poisson analysis.

Taken together, these analyses will inform the relevance of inoculum origin and availability to seedlings for their ability to cope with drought conditions and whether there is a direct relationship between mycorrhizal infection status, before or during drought, and the severity of the stress response.


Cortaña
360
Reicher
Austin
0
0.05
0.1
Kilometers
Reicher Inocula Collection Sites
Geographic Coordinate System: WGS 1984
Author: Silas Jenkins
Collection Site Type
Juniper
Oak



Cortaña Inocula Collection Sites

Geographic Coordinate System: WGS 1984
Author: Silas Jenkins

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