Changes in Growth of *Aristida purpurea* Steud. (C_4, Poaceae, Fender's three awn) in the Presence of Arbuscular Mycorrhizal fungi and/or *Solenopsis invicta* Buren (red imported fire ant)

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[2] This manuscript was part of Mr. Engelken's M.S. thesis. Unfortunately, he passed away before it could be published. We are publishing it in his honor. In addition, the thesis document has been edited, references have been updated and the thesis document has been reduced in size. The original document is available at the University of Texas at San Antonio Library.

ABSTRACT

Biomass and percent mycorrhizal infection of roots of *Aristida purpurea* Steud. (C_4 grass, Fender's three awn) changed in the presence of mycorrhizal fungi and/or the red imported fire ant (*Solenopsis invicta* Buren). *Aristida purpurea* dry mass produced and pattern of arbuscular mycorrhizal infection in the roots were examined in heat-sterilized and unsterile soil as well as in various stratified soils. The effectiveness of *S. invicta* as a fungal vector was correspondingly studied. Growth in heat-sterilized soil confirmed that *A. purpurea* is an obligate mycotroph. Percent mycorrhizal infection in non-sterile soil decreased with soil depth despite a uniform distribution of mycorrhizal propagules. Soil stratification altered the pattern of mycorrhizal infection, yet total plant biomass increased when infected roots were able to grow into sterile soil. Growth of *A. purpurea* appeared to be unaffected by delayed initial infection by mycorrhizal fungi as long as upper sterile soil was not more than 20 cm deep. *Solenopsis invicta* does not appear to be a vector of fungal spores and effects of *S. invicta* are subtle and elusive but can seemingly change soil biotic factors so positive growth of *A. purpurea* is maintained. Published on-line www.phytologia.org *Phytologia 101(4): 231-252 (Dec 21, 2019).* ISSN 030319430.

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Both biotic and abiotic factors can affect the growth of plants and ultimately the species composition of communities as well as the rate of community succession (Grace and Tilman 1990; Keddy 2017). There are many biotic factors including symbioses that can affect plant communities and some suggest that over 80% of all terrestrial species have mycorrhizal fungi as symbionts (Smith and Read 2008; Pagano 2012; Willis et al. 2013; Pagano and Grupa 2016). Discovering how these factors interact to influence the growth and distribution of plants is a primary goal of plant ecology (Begon et al. 2006; Keddy 2017).

Mycorrhizae have received attention for their role in affecting plant community composition and roughly 95% of terrestrial plant families have some members known to have mycorrhizal associations (Trappe 1987; Newman 1988; Bush 2008). Mycorrhizae are well known to increase plant growth especially in nutrient poor soils (Gianinazzi 1991; Van Auken and Brown 1998; Van Auken and Fredrick 2006; Bush 2008; Willis et al. 2013; Keddy 2017). Many pines and some orchids, are obligatorily dependent upon mycorrhizae for normal growth and development (Allaby 1992; Keddy 2017). Because mycorrhizae are so prevalent and their potential effects on plant growth so dramatic, it is critical that factors affecting mycorrhizae be examined to determine if relationships are commensal, mutualistic or parasitic.

A symbiotic relationship between the roots of a host plant and a specific soil fungus is a mycorrhizal association (Zak 1964). The presence of mycorrhizae in fossils suggests that this relationship has been coevolving since the Devonian 359-419 million years ago (Gianinazzi 1991). Mycorrhizae are usually mutualistic, absorbing and translocating nutrients (primarily phosphate) to the plant and getting carbohydrates in return (Hayman 1982; Gianinazzi-Pearson and Gianinazzi 1983; Willis 2013).

Mycorrhizae are either ectomycorrhizal (hypha growing between cortical cells) or endomycorrhizae (hypha penetrate root cells) (Peterson and Bonfante 1994; Smith et al. 1994) with ecto's usually associated with roots of woody plants and endo's more typical of non-woody species (Brundrett 1991; Francis and Read 1994). The most prevalent endomycorrhizae are the arbuscular mycorrhizae (Abbott and Gazey 1994) which develop arbuscules where nutrients are exchanged for carbohydrates (Marschner and Dell 1994; Smith et al. 1994; Van Auken and Brown 1998; Van Auken and Fredrick 2006; Bush 2008; Willis et al. 2013; Keddy 2017).

Mycorrhizal propagules are usually most abundant in the upper 50 cm of soil and their numbers decrease with increasing soil depth with few to none occurring at depths below approximately 100 cm (Smith 1978; Zajek et al. 1986). These mycorrhizal fungi have a low degree of host specificity, thus infecting a variety of plants that may lead to the establishment of a network of hyphal interconnections between plants (Read et al. 1985; Francis and Read 1994; Bush 2008; Willis et al. 2013; Keddy 2017). Host dependence upon mycorrhizae can be facultative or obligate but obligate species cannot survive to maturity without mycorrhizae (Miller 1987).

Mycorrhizal dependence was thought related to root morphology (Baylis 1975; Hetrick et al.1990). It has been suggested that C_3 grasses with more branched root systems are less reliant on mycorrhizal associations than C_4 grasses or forbes with coarser, less branched root systems. However, evidence suggested that biomass production in warm-season, C_4 grasses tended to be mycorrhizal dependent while cool-season, C_3 grasses responded less vigorously to mycorrhizae (Hetrick et al. 1988). This has also been attributed to temperature effects (Bentivenga and Hetrick 1992) because C_3 grasses are more common in cooler regions where mycorrhizae would suffer from lower metabolic activity.

Mycorrhizal associations are finely balanced with phenological and edaphic factors known to strongly influence the efficacy of mycorrhizal symbiosis (Brundrett 1991). Of these factors, soil organisms in particular, can influence the occurrence and effectiveness of mycorrhizae. Small mammals (Warner et al. 1987), earthworms (Mcilveen and Cole 1976), and macroarthropods (Visser 1985) have been shown to actively disperse mycorrhizal inoculum altering the distribution and density of propagules (Willis et al. 2013). Sow bugs and millipedes can act as effective vectors by ingesting and dispersing infective mycorrhizal inoculum (Rabatin and Stinner 1988; Wills and Landis 2018).

Because of the widespread abundance of mycorrhizal fungi in soils, they are thought to be an important food source for soil organisms, including nematodes (Hussey and Roncadori 1981) and springtails (Warnock et al. 1982). These soil organisms have been shown to reduce the yield of mycorrhizal plants by grazing on external mycelium. Thus, soil microorganisms may enhance (Fitter and Garbaye 1994), reduce (Hetrick et al. 1990), or not effect (Garbaye 1991) the efficacy of mycorrhizae depending on environmental conditions.

Two insect groups, termites and ants, have long been known for physically modifying soil through selecting, transporting, and rearranging soil particles (Lobry de Bruyn and Conacher 1990), but their potential as vectors for the dispersal of mycorrhizal fungi is not completely known. In addition, the importance of ants in grasslands is often unnoticed and the conversion of over 90% of North American grasslands and savannas to agriculture has put many prairie species at risk of extinction (Wills and Landis 2018). Also, these conversions have changed habitat conditions making many areas susceptible to invasion or encroachment by non-native species (Wills and Landis 2018). Interaction between ants and other species is well known, but complex (Del Toro et al. 2012) and

can alter diversity and community structure (Dostál 2007). Various ant species often reduce predator populations and even the density of parasitoids (Sanders and Van Veen 2011).

A species of ant relatively new to the southern United States including Texas is the red imported fire ant *Solenopsis invicta* Buren (Cook et al. 2016). It arrived in the United states in approximately 1939 and in Texas in 1956 (MacKenzie et al. 2019). There are numerous papers dealing with *S. invicta* management and control (see Woolfolk et al. 2016; MacKenzie et al. 2019; Qin et al. 2019), but little is known of any effects on plant roots or mycorrhizae associated with plant roots. The harvester ant, *Pogonomyrmex occidentalis,* has been shown to assist in the establishment of mutualistic mycorrhizal associations (Freise and Allen 1993; Snyder et al. 2002). However, this is currently unknown for the red imported fire ant. *Solenopsis invicta* has been shown to reduce populations of beneficial predators by as much as 50% (Eubanks et al. 2002).

Solenopsis invicta has continued to increase in abundance and range in North America, but there have been few studies concerning their effects on soil modification, soil organisms, and associated plants (Vinson and Sorensen 1986; Snyder et al. 2002). *Solenopsis invicta* ants are known to feed on the roots of some crop species (Sittle et al. 1983). Concentrations of organic matter and certain mineral nutrients were higher at the surface of *S. invicta* mounds than in the surrounding soil surface. At 15-20 cm depth, only organic matter and potassium were significantly higher in the mounds and average depth of ant activity was 30.0 cm (Lockaby and Adams 1985). *Solenopsis invicta* is known to out compete the native fire ant (*Solenopsis geminate* F.) and replace native colonies at a ratio of 6:1 (Porter et al.1988).

It is possible that pedoturbation by fire ants could result in dispersal of mycorrhizal propagules and changes in plant community structure because they can excavate as much as 1600 kg/ha/yr. But, many of these changes or effects are difficult to detect or observe because they occur below ground (Sanders et al. 2011; Wells and Landis 2018). Nest surface area is small, but below ground area is more extensive making patches or mosaics available for colonization by new and different plant species (Lobry de Bruyn 1999; Boultin and Amberman 2006; Drager et al. 2016). These changes in soil may change microbial activity in and around a nest including actives of soil mycorrhizal fungi, potentially increasing the presence of additional spores and root contact (Dauber et al. 2001, 2008).

PURPOSES

The first purpose of this study was to confirm that growth of the C_4 grass *Aristida purpurea* was mycorrhizal dependent. Second, there was a pattern of mycorrhizal infection and biomass allocation of *A. purpurea* through the vertical soil rhizosphere. Third, could delaying initial infection reduce potential early inhibitory effects of the fungi on the grass? Fourth, what was the potential of *Solenopsis invicta* to act as a vector for spreading mycorrhizal propagules throughout the vertical soil profile?

METHODS

General methods used for all experiments are presented first. All experiments utilized the top 20 cm of a Patrick-series Mollisol. The soil is classified as a clayey-over sandy, carbonatic-thermic, typic calciustoll (Taylor et al. 1966). It was collected near the University of Texas at San Antonio campus in Bexar County, Texas (29°35'N, 98° 40'W). Surface vegetation and litter was removed and the soil sieved through a 6.4 mm mesh sieve, air dried and mixed. A previous soil analysis showed a mean phosphorous content of 12 mg/kg, mean nitrogen content of 1 mg/kg, mean potassium content of 159 mg/ kg, (available form), and a moderately alkaline soil with a pH of 8.4 (Van Auken and Brown 1998). Sterilized soil was sieved, autoclaved at 121°C and 15 ATM for one hr with a 15 min drying period.

In all experiments, the C_4 grass *Aristida purpurea* Steud. (Fender's three awn) was grown from seed in a greenhouse. *Aristida purpurea* is a tufted perennial found on rocky or sandy slopes or in disturbances and is locally abundant in central Texas (Correll and Johnston 1979; USDA-SCS 2019). Seeds of *A. purpurea* were collected from near the campus of the University of Texas at San Antonio in

the spring of 1989 and were stored dry at 4°C until used. For each pot, ten seeds were sown initially by inserting them in the soil such that the awns were the only portions of the seed exposed above the soil surface. The pots were covered with a shade cloth to prevent drying of the surface during germination. After one week, the shade cloth was removed and density was reduced to three plants per pot. Plants were watered as needed, usually every two days, with approximately 200 ml of deionized water to maintain field capacity. Pot locations were haphazardly adjusted weekly to insure equal light exposure in the greenhouse. Photosynthetically active photon flux density (PPFD 400-700 nm) in the greenhouse at solar noon, October 22, 1993, was 562 ± 135 µmol/m^2/s (mean ± SD) or 36% of outside PPFD that was 1542 ± 18 µmol/m^2/s. Light intensity was measured with a Li-Cor ® Li-1000 Data Logger with an integrating quantum sensor.

After fourteen weeks, plants were harvested and dry mass and percent infection measured. The above-ground shoots were removed by cutting at the soil surface, oven dried at 90° C for 48 hr and dry mass measured per pot. Roots were washed with tap water to remove soil. Wet root weights were recorded and a small root sample was obtained from three areas of the root for a total of approximately 0.3 g of sample. These samples were stained to determine percent mycorrhizal infection. The remaining roots were dried at 90° C for one week, and their dry mass measured. Ash-free root dry mass, minus the sample for staining, was obtained by ashing the dried roots at 700° C for 3 hr (Bohm 1979). The ash-free dry mass of the stained root samples was calculated by using a regression analysis of the ash-free dry mass versus final wet root weight. This gave the equation of a line which was used to estimate the ash-free dry mass of the sample from its wet weight. The equation for the line was y = 0.119x + 0.000 (R^2 = 0.96, P=0.001). Total root dry mass was determined by summing the ash-free dry mass with the estimated dry mass of the root sample used for staining.

Root samples in all experiments were stained for mycorrhizal detection using the trypan blue technique (Phillips and Hayman 1970). Fresh root samples were washed, cut into 1 cm segments, placed in 10% potassium hydroxide and heated for twenty minutes at 90°C. After clearing, root samples were rinsed, then placed in a mild bleach to further clear the roots. The bleach was removed and the root samples soaked in acidified water for ten minutes. After water removal, samples were stained in 0.05% trypan blue, de-stained and stored in 85% lactic acid until they were examined.

Root infections were determined using the modified gridline method (Newman 1966; Giovannetti and Mosse 1980; Van Auken and Brown 1998; Van Auken and Freidrich 2006). The lid from a 9.5 cm petri dish was used with gridlines at 0.5 cm intervals. Points where a root intersected a line at nearly a perpendicular angle were examined and only the area of the root over the line was considered when determining infection. The microscope was focused through the entire root and if any arbuscules or vesicles (but not coils or hyphae) were found, the root segment was considered infected. One hundred and fifty root-gridline intersections were examined for each sample and the percent infection calculated.

Aristida purpurea biomass and infection were determined in response to benomyl application in heat sterilized and non-sterilized soil. This was done to demonstrate that *A. purpurea* growth in sterile soil resulted from the mycorrhizal fungi being rendered inviable as opposed to the plant growth being a consequence of changes in other soil factors following the autoclaving procedure. To do so, *A. purpurea* plants were grown in sterile and unsterile soil in the presence and absence of the fungicide benomyl.

Ten 15 cm diameter by 15 cm deep pots were filled with 1400 g of dried non-sterile Patrick-series soil while another ten pots were filled with the same mass of soil heat sterilized by autoclaving. Five pots of each treatment were selected and a 100 ml suspension of benomyl was thoroughly mixed with soil in the pots. The benomyl concentration was 45 mg/kg in the soil. Benomyl is insoluble in water and was added to the soil as a suspension then thoroughly mixed.

Ten seeds of *A. purpurea* were sown per pot on July 27, 1994. The density was reduced to three plants per pot after one week. During the sixth week of growth, another 100 ml of a benomyl

suspension was added to the surface of the pots bringing the total concentration of benomyl to 90 mg/kg of soil. After 17 weeks, all plants were harvested on November 30, 1994. Shoot dry mass and ash-free root dry mass were measured along with percent infection of the roots. All dry mass measures were square root transformed and the percent infection was arc sine transformed to reduce variance (Lindman 1992). An *F*-test was used and is robust when the assumption of equal variances is violated as long as the samples are equal (Lindman 1992). SAS was used to conduct a two-way analysis of variance on shoot, root and total dry mass as well as percent infection with soil sterilization and benomyl treatment as main effects along with the interaction of these factors.

Non-nutrient agar was added to some pots to separate soil treatments. Biomass and infection of *A. purpurea* was examined in response to the addition of non-nutritive agar in sterile and non-sterile soil to demonstrate that it did not stimulate plant growth. Seven treatments were prepared in which sieved Patrick-series soil was mixed with increasing amounts of agar. The treatments were 0.0 g, 0.28 g, 0.56 g, 0.84 g, 1.13 g, 1.69 g and 2.25 g of undissolved agar mixed with 3400 g of soil and then added to 10.2 cm diameter x 40 cm tall pots constructed from PVC pipe. There were three replicates per treatment and care was taken to randomize soil/agar additions across treatments. Seeds were sown on March 9, 1994 and plants were harvested after a 14-week growing period. Shoot dry mass, root ash-free dry mass and percent mycorrhizal infection were measured. SAS was used for a one-way analysis of variance of response variables. Scheffe's multiple comparison tests were used to compare treatment means.

The response of *A. purpurea* to non-sterilized and heat sterilized soil as well as three non-sterile/sterile and three sterile/non-sterile stratified soil treatments was next examined. Shoot dry mass, root dry mass, along with total dry mass, and percent infection of the roots, every 10 cm in soil depth, was examined to describe the pattern of mycorrhizal infection and allocation of biomass throughout the vertical rhizosphere. Forty total pots, 10.2 cm diameter x 40 cm tall, were used, replication was 5 pots/treatment. Each pot was filled with 3400 g total of Patrick-series soil that was non-sterilized or heat sterilized. Some pots had different layers of soil stratified as non-sterile on top and sterile on the bottom (+/-), or the reverse (-/+). Various layers were prevented from mixing by placing a 1 cm thick layer of sterile, non-nutritive, agar (75 ml of a 7.5 g per 100 ml distilled H_20 mixture) between them. Each layer contained 850 g of the desired soil (four total) and was added with care taken to randomize the soil treatments. Pots were divided into four vertical segments of 10 cm each. After addition and solidification of the agar layer, the next layer of soil was added to fill the pot to its total of 3400 g. Five pots were setup for each of the following treatments: 40 cm non-sterile (+), 40 cm sterile (-), 20 cm-/20 cm+, 20 cm+/20 cm-, 30 cm-/10 cm+, 30 cm+/10 cm-, 10 cm-/30 cm+, and 10 cm+/30 cm-.

On July 29, 1993 *A. purpurea* seeds were sown as previously described. Plants were allowed to grow for 14 weeks and were harvested during a five day period from November 18, to November 22, 1993. One replicate per treatment was harvested each day. Roots were removed from the pots by flooding the pot with water, then inverting the pot to allow the soil and roots to slide out as a column. The column was then cut into 10 cm segments and the roots washed. A one-way analysis of variance was conducted to determine if the soil treatments affected the dry mass of the plants and the percent fungal infection. All response variables were transformed as previously indicated. Scheffe's multiple comparison test was used to determine significant differences because of its general conservatism.

The next experiment examined the biomass and root infection of *A. purpurea* in the presence of mycorrhizal fungi, soil stratification and the presence of a potential fungal propagule vector *Solenopsis invicta*, the red imported fire ant. Response variables were measured every 10 cm in soil depth. Sieved Patrick-series soil was non-sterile or sterile and placed in 10.2 cm diameter x 40 cm tall pots. Treatments consisted of five pots with all sterile (-) and five with all unsterile soil (+). Stratified treatments were set up with sterilized soil in the top 20 cm and non-sterilized soil in the bottom 20 cm of soil (20-/20+) as well as the reverse (20+/20-). A 1 cm thick agar layer was placed in each pot at a depth of 20 cm to prevent soil mixing. The same was done to the pots with all sterile

and all unsterile soil. Each treatment had five replicates. The four soil treatments were duplicated to examine potential effects of *S. invicta*. Thus, a total of forty pots were used, twenty with ants and twenty pots without ants.

Ten *Aristida purpurea* seeds were sown and thinned as previously noted. *Solenopsis invicta* were collected from a single colony located on the University of Texas at San Antonio campus. The mass equivalent of one thousand fire ant workers from a variety of castes was added to each of the ant treatment pots on April 17, 1994, three-and-half weeks after initial seed germination. At the same time, two queens and two hundred larvae, at various stages of development, from the same colony were added to these pots. The mean mass of one thousand S. *invicta* was 0.153 ± 0.012 g. This meant that on average each pot received 1000 ±80 ants.

Pots were modified to contain the *S. invicta* using thin, clear plastic, circular trays, 15.25 cm in diameter, with a circular hole cut in the center equal to the diameter of the pot. The tray was glued to the outside of the pot with silicone caulk. The inside wall of the tray was treated with a layer of fluon which prevented the ants from escaping. As the grass grew, some leaves were prostrate over the side of the tray, allowing the ants to escape. A cylinder, 9 cm in diameter by 7 cm tall, was made from 1.27 cm hardware cloth and placed on the soil surface. Pots without ants received the same modifications. The *S. invicta* ants were given water and a diet of a mixture of eggs, cooked hamburger, gelatin, sugar, salt, and a drop of vitamins (Banks 1981). Throughout the experiment, ant mortality was measured weekly by removing and counting the number of dead ants.

After 16 weeks, the live *S. invicta* adults and larvae were removed from the pots and the plants harvested over an eight-day period from July 2, to July 11. To remove the ants, a small hole was drilled in the bottom of the pot allowing it to be filled with water from the bottom to the top. Water was slowly added and the ants and larvae that were brought to the top were counted and removed. The shoots of the grass were cut at the soil surface and their dry mass measured. Root ash-free dry mass and percent infection was measured every 10 cm of pot depth.

A one-way analysis of variance was conducted on total ant mortality at the end of the experiment, total number of ants at harvest, the sum of total mortality and number of ants at harvest, and total number of larvae at harvest. Scheffe's multiple comparison test was used to separate treatment means. Transformations were as previously indicated. A two-way analysis of variance was performed with soil and *S. invicta* treatments as main effects along with their interaction. Student-Newman-Keuls multiple comparison test was used to separate treatment response variables (Einot and Gabriel 1975).

RESULTS

Biomass and percent root infection of *Aristida purpurea* were significantly reduced by soil heat sterilization and benomyl treatment (two-way ANOVA, $P < 0.0001$ for all). In non-sterile soil, mean total plant dry mass without benomyl was 3.01 g and with benomyl it was 0.05 g. Mean percent infection in non-sterile soil without benomyl was 27.5 % and with benomyl it was 2.0 %. Mean total *A. purpurea* dry mass in heat sterile soil was 0.06 g without benomyl and 0.04 g with benomyl, while the mean percent infection in heat sterile soil was 0.0 % with or without benomyl. Trends for shoot and root dry mass were the same as for total dry mass.

Addition of non-nutrient agar did not significantly affect dry mass or percent infection of *A. purpurea* in non-heat sterilized soil (one-way ANOVA, $P > 0.05$ for all, Figure 1). Percent infection ranged from 28.7 % to 43.6 % and was not significantly different. Shoot dry mass was approximately twice as high as the root

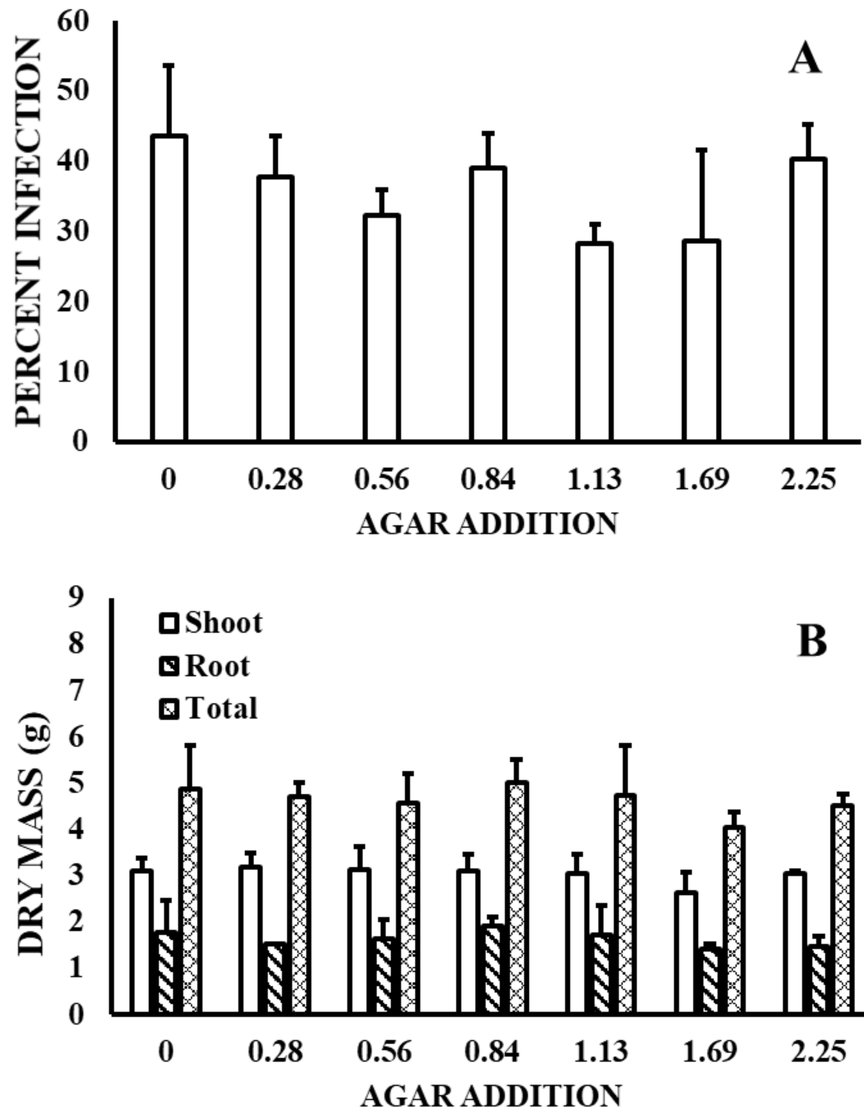

Figure 1. Mean percent infection (A) and mean root, shoot and total dry mass (B) of *Aristida purpurea* for each non-nutrient agar addition in grams in non-sterile soil. The line at the top of each bar is + one standard deviation of the mean.

dry mass and total dry mass ranged from 4.04 to 5.01 g/pot. The effects of addition of non-nutritive agar on the dry mass and percent infection of *A. purpurea* in heat sterilized soil showed that shoot dry mass was significantly affected by agar addition ($P < 0.05$), but values and differences were small with high variability, and the root and total dry mass values were not significantly affected by agar addition ($P > 0.05$ for both, not shown). Percent root infection for all was zero.

Overall results of the effects of various soil stratifications on the response variables of *A. purpurea* showed that shoot, root, total dry mass and percent root infection measurements were all significantly affected by the various soil stratifications (one-way ANOVA, $P < 0.0001$ for all). Based on Scheffe's multiple comparison test, *A. purpurea* response variables were all significantly reduced in plants grown in sterile soil (all-, $P< 0.05$) compared to those in the non-sterile control (all+). Mean shoot, root and total dry mass for plants grown in sterile soil were 0.04 g, 0.04 g, and 0.08 g,

respectively (Table1). In non-sterile soil, mean dry masses were 1.82 g, 2.52 g and 4.34 g, respectively (Table 1). Mean percent infection per pot was reduced from 13.1 % in the non-sterile soil control to 1.7 % in sterile soil (Table 1).

Table 1. Mean shoot, root, total dry mass and percent infection of *A. purpurea* for each soil treatment. A – indicates the soil was sterilized while a + indicates non-sterile. Dry mass means are presented in grams. Values for a treatment in a column with the same letter are not significantly different (Scheffe's multiple comparison test, P > 0.05).

Soil Treatment	MEAN DRY MASS (g)			% Root Infection
	Shoot	Root	Total	
ALL -	0.04b	0.04c	0.08c	1.7c
ALL +	1.82a	2.52a	4.34a	13.1a
20+/20-	2.46a	3.63a	6.09ab	11.4ab
20-/20+	1.70a	2.51a	4.21ab	6.3abc
30+/10-	2.47a	3.28a	5.76ab	13.9a
30-/10+	0.14b	0.23b	0.37c	5.1bc
10+/30-	2.06a	3.72a	5.78ab	13.0a
10-/30+	2.47a	4.28a	6.75a	12.9a

The Scheffe multiple comparison test revealed that none of the stratified soil treatment significantly increased mean total dry mass above that of the non-sterile control (all+, $P > 0.05$, Table 1). Mean total dry mass in the 10-/30+ treatment was 6.75 g compared to 4.34 g for the non-sterile control, but not significantly different (Table 1). In all soil treatments, except the sterile treatment (all- and the 30-/10+), mean root dry mass exceeded mean shoot dry mass from 1.33 to 3.1 times (Table 1).

Vertical soil profiles of the rhizosphere showed where the root was infected and how that modified dry mass. For *A. purpurea* plants grown in sterile soil (all-) the vertical pattern of infection was low compared to plants in non-sterile soil (all+, Figure 2A). In the non-sterile (all+) soil, infection decreased gradually and uniformly from 18.3% in the top 10 cm section to 13.6% in the 30 cm depth followed by a rapid decline to 4.9% in the 40 cm bottom section (Figure 2A). Mean percent infection for the entire pot was 13.1%. In the 20+/20- soil treatment, the pattern of infection was similar to the non-sterile treatment. However, percent infection in the 20 cm section and the 30 cm section (Figure 2A) were equal but the 20 cm section of root was in the non-sterile soil while the 30 cm section was in sterile soil. Infection in the 40 cm segment was 2.9%. For plants in the 20-/20+ treatment, infection was reduced but highest in the 30 cm section of the pot at 10.5% (Figure 2A). The roots in the 10 cm and 20 cm sections (in sterile soil) were equally infected but higher than those in all segments of the all sterile treatment (Figure 2A).

For plants in the 30+/10- treatment, the vertical pattern of infection was similar to that in non-sterile soil (all+), except that there was an increase in percent infection from the 30 cm section (unsterile soil) to the 40 cm section (sterile soil, Figure 2B). In the 30-/10+ treatment, percent infection was generally lower, but increased in the 40 cm section with high variance (Figure 2B). The pattern of infection in the 10+/30- treatment was asymmetrical in that the 10 and 20 cm root sections were much

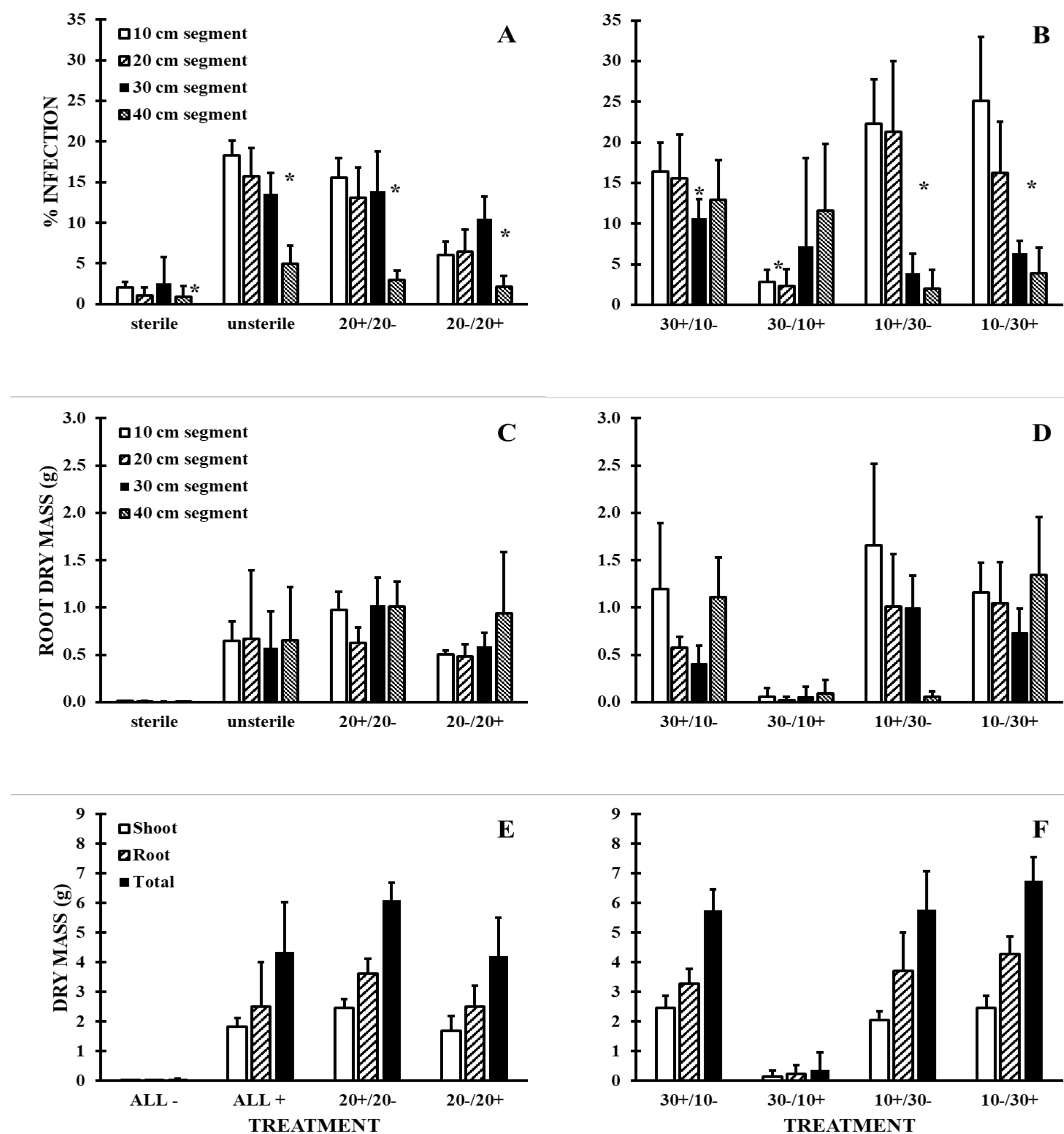

Figure 2. Mean percent infection (A, B), root dry mass for each 10 cm pot segment (C, D), and mean total shoot, root, and total dry mass (E, F) for *Aristida purpurea* in each soil treatment. Mean per pot infection = (*) and line at top of bar is +1 SD. A – is sterile and + is non-sterile soil.

more highly infected than the 30 and 40 cm sections (~21.0% vs. ~3.0%, Figure 2B). The 10-/30+ treatment was similar to the previous treatment but showed a more progressive decrease in percent infection from 25.1 % in the 10 cm root section to 3.9 % in the 40 cm section (Figure 2B). Percent infection in the -10 cm root section was highest among all root sections from every treatment and occurred in a region of sterile soil (Figure 2B).

Allocation of root dry mass throughout the vertical rhizosphere in sterile soil (all-) was very low (Figure 2C). In non-sterile soil (all+) root dry mass was similar in each section. In the 20+/20- soil treatment, root dry mass was higher but allocated almost equally in all root sections (Figure 2C). For plants in the 20-/20+ treatment, root dry mass increased slightly with depth in the non-sterile soil (Figure 2C). Root dry mass in the 30+/10- soil treatment, was higher in the 10 cm and 40 cm root

sections when compared with the 20 cm and 30 cm sections (Figure 2D). Root dry mass in the 30-/10+ treatment, was quite low in each section (Figure 2D). In the 10+/30- soil treatment, root dry mass in the 10 cm section was highest and then dropped with increasing depth and there was very little root dry mass in the deepest soil segment that was sterile (Figure 2D). For plants in the 10-/30+ treatment, root dry mass dropped from the 10 to the 30 cm section but increased in the 40 cm non-sterile soil section (Figure 2D). Total dry mass was low in the all- segments of the sterile soil treatment (Figure 2E). Total dry mass was higher in all treatments except the 30-/10+ and was highest in the 10-/30+ soil treatment.

Solenopsis invicta was examined as a potential fungal vector in pots with *A. purpurea.* Total mortality of *S. invicta* differed significantly with soil treatment (one-way ANOVA, $P < 0.05$, Figure 3). However, the number of larvae at harvest, the number of adults at harvest and the sum of live and dead *S. invicta* at harvest were not significantly different in the soil treatments (one-way ANOVA, $P > 0.05$, Figure 3A, C and D), but total mortality was significantly different (one-way ANOVA, $P < 0.05$, Figure 3B). Mean total *S. invicta* mortality was significantly lower in the non-sterile (all+) and 20+/20- soil treatments compared to the sterile treatment (all-) but not significantly different in the 20-/20+ treatment (Scheffe multiple comparison test, $P > 0.05$). Mean total mortality in the sterile soil treatment was 477.6 and 380.6 in the 20-/20+ treatment, while it was 237.4 in the non-sterile treatment and 202.4 in the 20+/20- treatment. Even though approximately one thousand *S. invicta* adults were added to each pot initially, at the end of the experiment mean sum of total ants including mortalities was 583-887 ants for all soil treatments with no significant differences.

Overall, *Aristida purpurea* shoot and total dry mass were significantly affected by the presence of *S. invicta* (two-way ANOVA, $P < 0.05$). However, root dry mass and percent infection of *A. purpurea* were not significantly affected by *S. invicta* (two-way ANOVA, $P > 0.05$). Soil treatment had a significant effect on *A. purpurea* response variables (two-way ANOVA, $P < 0.0001$). The two-way interaction of *S. invicta* and soil treatment was significant only for shoot dry mass meaning that *A. purpurea* shoot dry mass increased significantly with *S. invicta* but only in non-sterile soil (two-way ANOVA, $P < 0.05$). Student-Newman-Keuls groupings of shoot, root and total dry mass were significantly lower in the sterile (all-) and 20-/20+ soil treatments compared to the non-sterile (all+) and 20+/20- soil treatments (Table 2). Also, the percent infection per pot was significantly lower in sterile soil (all-) compared to the 20-/20+ soil treatment (Table 2). Percent infection in the 20-/20+ soil treatment was significantly lower than that in the non-sterile (all+) and 20+/20- soil treatments (Table 2, Figure 4). The only significant difference in shoot and total *A. purpurea* dry mass in the presence of *S. invicta* occurred in non-sterile soil (all+) where shoot and total dry mass increased by 67.3% and 49.3%, respectively (Table 2, Figure 4). With *S. invicta* present in the 20+/20- soil treatment, shoot and total dry mass increased by 19.0% and 16.3%, respectively, but not significantly (Table 2). In the sterile (all-) and 20-/20+ soil treatments, shoot and total *A. purpurea* dry mass decreased with *S. invicta* compared to without *S. invicta*, but was not significantly different (Table 2). It is also interesting to note that without *S. invicta* present, Student- Newman-Keuls groupings show that shoot and total dry mass of *A. purpurea* were significantly higher in the 20+/20- soil treatment compared to the unsterile soil treatment even though percent infection was lower for the 20+/20- treatment, but not significantly lower.

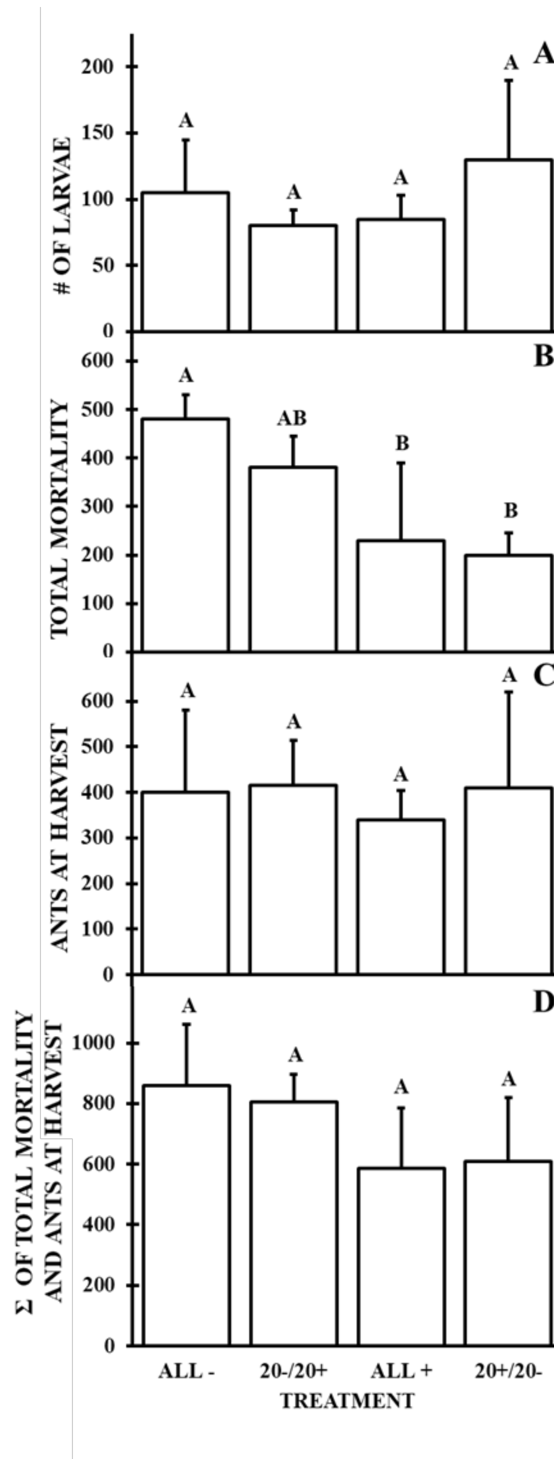

Figure 3. Mean number of larva (A), total mortality (B), adult ants at harvest (C), and sum of total mortality and adult ants at harvest (D). Line at the top of a bar is 1 SD. Means with the same letter at the top are not significantly different (Scheffe's multiple comparison).

Table 2. Mean shoot, root, and total dry mass and mean percent infection per pot of *A. purpurea* for each soil and ant treatment. Means for a treatment in a column with the same letter possess means which are not significantly different when tested with Student-Newman-Keuls multiple comparison test. A - indicates sterile soil while + indicates unsterile soil.

Soil Treatment	Ant Treatment	MEAN DRY MASS (g) Shoot	Root	Total	% Root Infection
ALL -	w/ ants	0.03c	0.02c	0.05c	0.22c
	w/o ants	0.04c	0.02c	0.06c	0.22c
20-/20+	w/ ants	0.12c	0.10c	0.22c	4.6b
	w/o ants	0.19c	0.18c	0.37c	7.5b
ALL+	w/ ants	2.81a	2.7ab	5.51a	35.0a
	w/o ants	1.68b	2.03b	3.71b	40.8a
20+/20-	w/ ants	2.94a	3.13a	6.07a	26.4a
	w/o ants	2.47a	2.76ab	5.23a	26.5a

Examination of the pattern of percent mycorrhizal infection throughout the vertical rhizosphere showed that *A. purpurea* plants grown in sterile (all-) soil, with and without *S. invicta,* had very low levels of root infection ranging from 0.0% to 4.5% for all root sections (Figure 5A). In the 20-/20+ soil treatments with *S. invicta,* percent infections were low and somewhat uniform throughout the root ranging from 3.3% in the 30 cm section to 8.0% in the 20 cm section (Figure 5A). Without *S. invicta,* percent infection increased to 16.2% and 13.8% in the 20 cm and 30 cm (heat sterilized) root sections although variation was high (±28.1% and ±16.9% respectively, Figure 5A). The non-sterile (all+) soil treatment was high, with and without *S invicta*, and showed a similar decrease in percent infection with increasing soil depth from about 70.0% to about 15.0% (Figure 5B). In the 20+/20- soil treatment, with and without *S. invicta*, percent infection decreased as soil depth increased with little variation between the two treatments. Regardless of ant treatment, percent infection for the 40 cm root segment in the 20+/20- soil treatment, ranged from 0.0% to 0.3% and was much lower than for the non-sterile treatment which ranged from 11.0% to 15.1 % (Figure 5B). There was little root dry mass in all soil sections in the vertical rhizosphere of *A. purpurea* plants grown in sterile soil (all- or 20-/20+) with or without *S. invicta* (Figure 5C). For *A. purpurea* plants in the non-sterile soil treatment (All+, 20+/20-), root dry mass allocation increased slightly with depth with and without *S. invicta*. In the 20+/20- soil treatment, root dry mass allocation was more uniform with *S. invicta* than without them (Figure 5D). All dry mass measured in the sterile soil treatments were very low (Figure 5E).

Total dry mass for the all+ treatments were significantly different with less dry mass when *S. invicta* was absent. In the 20+/20- total *A. purpurea* dry mass there were no significant difference with or without *S. invicta* but total dry mass was higher when ants were present (Figure 5F).

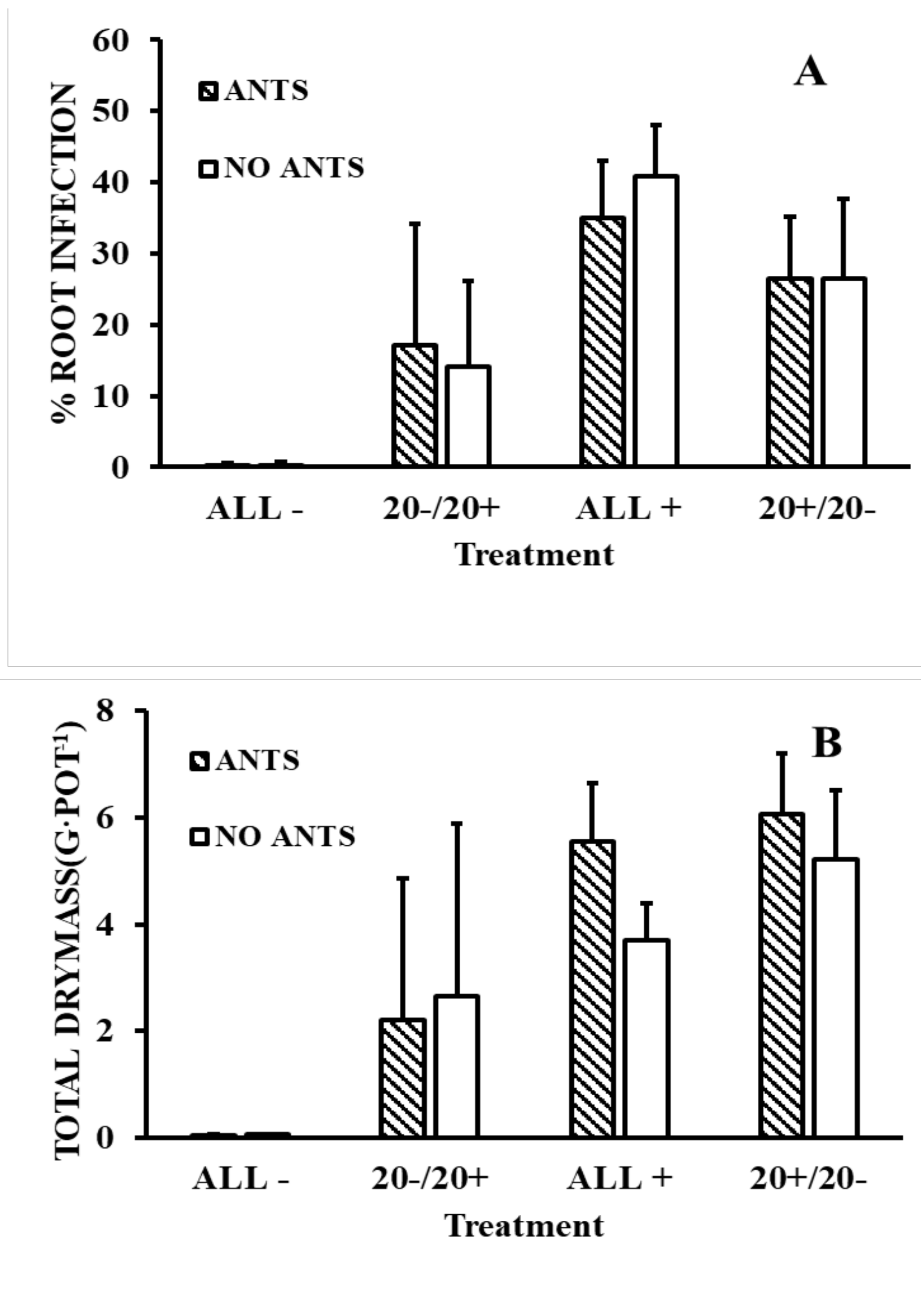

Figure 4. Mean percent infection per pot (A) and mean total dry mass per pot of *Aristida purpurea* for each soil and *Solenopsis invicta* treatment. The line at the top of a bar is + 1 SD. A – indicates sterile soil while a + is non-sterile.

DISCUSSION

Soils are complex and effects of abiotic and biotic soil factors on higher plants are difficult to sort out (Willis et al. 2013; Wills and Landis 2018). Of the biotic factors in the soils, arbuscular mycorrhizal fungi are particularly important to the growth and survival of most vascular plants. Over 80% of terrestrial plants are infected with AM fungi with 95% of terrestrial plant families having species with mycorrhizal associations including the Poaceae or grasses (Trappe 1987; Wang and Qui 2006; Bush 2008). This mutualistic association includes the fungal contribution to the higher plants' mineral nutrition especially the uptake of phosphorus and other limiting soil nutrients and the plant contributing carbohydrates to the fungus (Bolan 1991; Clark and Zeto 2000; Bush 2008; Leigh et al. 2008; Willis et al. 2013). Mycorrhizae are well-known to increase plant growth especially in nutrient poor soils (Gianinazzi 1991; Van Auken and Brown 1998; Van Auken and Fredrick 2006; Bush 2008; Willis et al. 2013; Keddy 2017). But, there are other ways that these fungi can alter the organization and even structure of various plant communities (Bush 2008; van der Heijden et al. 2008; Willis et al. 2013; Wills and Landis 2018).

Grassland plants such as *Aristida purpurea* and grassland or prairie ecosystems have been and should continue to be a focus of research on mycorrhizal associations. Grasslands support a high degree of diversity of both plants and animals (Wills and Landis 2018). In addition, over 90% of North American grasslands have been converted to other uses, mainly agriculture (Samson and Knopf 1994). *Aristida purpurea* is a C_4 warm season grass that seems to be early successional or a grass that uses or exploits disturbances (Gould 1975; Van Auken and Brown 1998). It is found at low to mid-elevations across the central United States to the Pacific Ocean, in the western provinces of Canada, and the northern states of Mexico (USDA-NRCS 2019). This species is one of many C_4 grasses that grow in disturbances caused by a number of different influences including heavy cattle grazing. The colonization of the roots of these grasses by mycorrhizal spores in the soil is necessary for their growth and could be assisted by various soil organisms including one or more native or introduced Hymenoptera, specifically some of the Formicidae: ants (Wills and Landis 2018). The role of ants in grasslands is important but not completely understood. They are considered ecosystem engineers and can influence the population size and diversity of various invertebrates, plants and soil microorganisms (Holldobler and Wilson 1990; Del Toro et al. 2012; Boulton and Amberman 2006; Sanders and van Veen 2011; Wills and Landis 2018).

We have confirmed that heat sterilized soil reduced *A. purpurea* growth to almost zero. Also, the addition of the fungicide benomyl to soil reduced growth of *A. purpurea* to almost zero, similar to a previous report (Van Auken and Brown 1998). This demonstrated that the cause of the reduction in dry mass was due to the reduction or elimination of viable mycorrhizal propagules by the fungicide and not as a consequence of other potential changes to the soil caused by temperature. Further evidence showed the fungicide benomyl caused the reduction of the percent infection of *A. purpurea* roots in benomyl treatment soil. The effect of the fungicide on the growth of *A. purpurea* was analogous to that of soil heat sterilization. Thus, fungicide or heat sterilization could be used to eliminate soil fungi, but the reduction of other soil organisms by heat sterilization did not affect *A. purpurea* dry mass production or growth. Objections raised by others that killing all other soil organisms including the fungi, would cause potential masking of mycorrhizal effects are not warranted (Stribley 1987). Neither mortality nor number of seeds produced was assessed in this experiment, but all plants grown in sterile soil survived but never produced seed, while those grown in unsterile soil produced seeds (Engelken 1995).

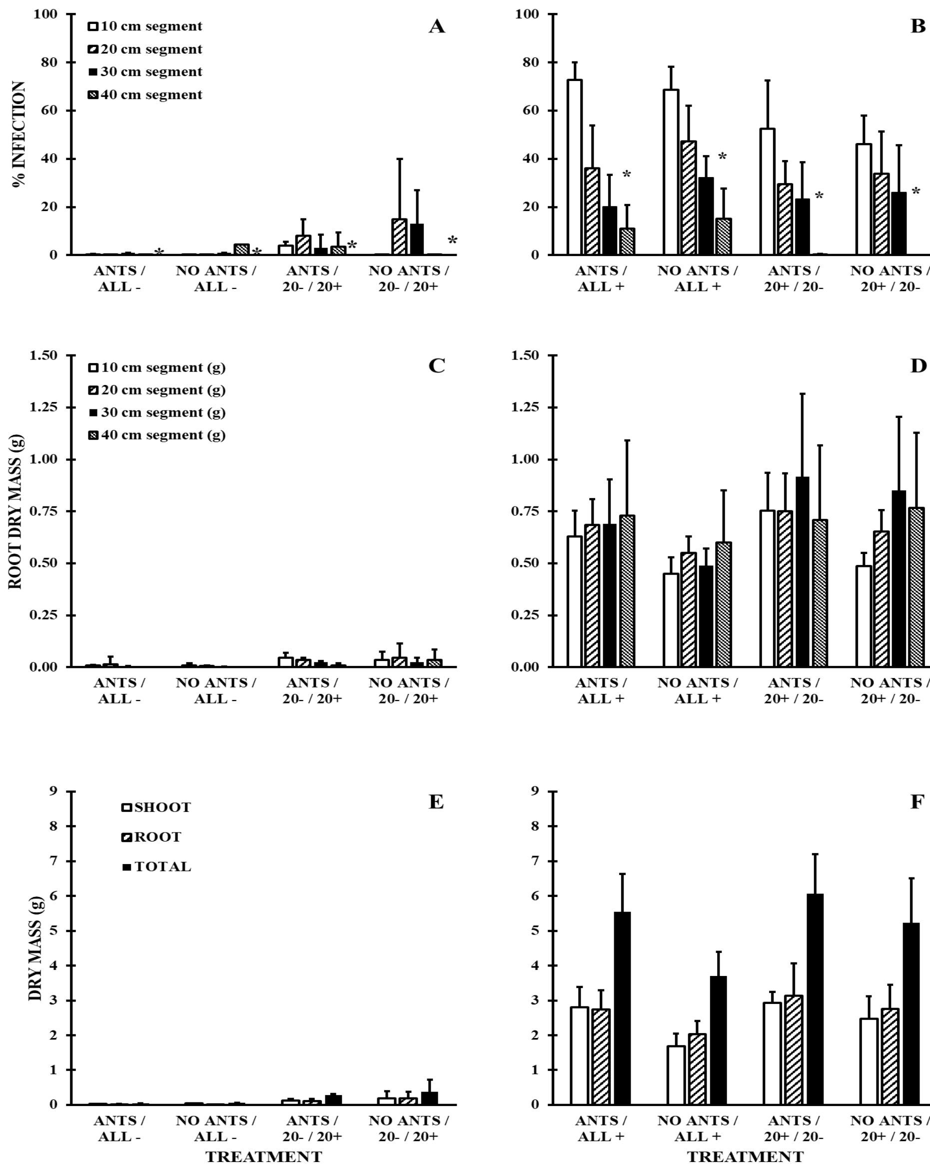

Figure 5. Mean *A. purpurea* percent infection (A, B) and root dry mass (C, D) per 10 cm pot section and total shoot, root and pot dry mass for each soil and *S. invicta* treatment (E,F). The line at the top of a bar is + 1 SD. Sterile soil is indicated by a - while non-sterile is indicated by a +.

Because *A. purpurea* grows in disturbances, we wanted to determine how much of a disturbance to the soil they could survive. Before we could do that, we had to determine how to spatially separate sterile (disturbed) and non-sterile (non-disturbed) soil, thus another experiment was required. We added increasing amounts of non-nutrient agar to pots with non-sterile soil and *A. purpurea* to see if root percent infection decreased and if plant dry mass increased due to a fertilizer effect by the addition of the agar (Willis et al. 2013). The addition of non-nutritive agar to the soil did not act as a fertilizer. For agar addition in sterile soil, there was a slight but significant increase in *A. purpurea* shoot dry mass, but this increase was so small as to be biologically unimportant and

potentially not an effect of the agar but an artifact. Generally, fertilizer addition results in a significant reduction in mycorrhizal infection because greater nutrient availability in the soil renders the mycorrhizal symbiosis superfluous (Menge et al. 1978). Therefore, if agar addition acted as an effective fertilizer, mycorrhizal infection would have been lower with increasing agar addition. This would also be coupled to increases in plant dry mass as reported by others (Owusu-Bennoah and Mosse 1979; Hijri et al. 2006; Willis et al. 2013). The results of the current study demonstrated that neither of these were the case.

When the upper layer of soil, top 0-30 cm, is severely disturbed, the mycorrhizal inoculum potential is considerably reduced (Jasper et al. 1979; Willis et al. 2013). The reduction could be short term, depending on degree of disturbance and local environmental conditions. Consequently, we examined the root infection and ability of *A. purpurea* to grow through a sterile layer of soil. Because *A. purpurea* is a species found in disturbances, it would have to start growth in disturbed surface soil. We examined the effect of various sterile/non-sterile soil stratifications on the growth and percent infection of *A. purpurea.* We showed that if the upper 20 cm of pot soil was sterile that root infection was reduced by approximately 83% and total dry mass was reduced by approximately 90%. In addition, results showed that when mycorrhizal fungi infected the roots of *A. purpurea*, and the roots grew into sterile soil, there was an increase in dry mass of the plant. The increase was mostly in the shoot. Increases in shoot:root ratios of mycorrhizal plants have been attributed to enhanced efficacy of the mycorrhizal association present (Marschner and Dell 1994). However, some studies with C_4 grasses have shown that soil microflora can negatively affect the mycorrhizal symbiosis (Daniels Hetrick et al.1988; Hetrick et al.1988), possibly by competing for nutrients, resulting in reduced plant dry mass and root infection. It appears that *A. purpurea* plants with roots that are infected with mycorrhizae in sterile soil avoid the antagonistic effects of other microorganisms and are more effective taking up nutrients than those plants grown in non-sterile soil. The roots in sterile soil may have become infected as external hyphae grow and extend along the length of the root from primary infection sites in non-sterile soil to secondary sites in sterile soil (Sanders and Sheikh 1983).

When looking at the pattern of mycorrhizal infection of *A. purpurea* roots in the vertical rhizosphere, percent infection decreased with increasing soil depth in unsterile soil even though propagule density was initially uniform throughout. This pattern of infection was seen with grasses in field experiments by others (Zajicek et al. 1986). It is also interesting to note even though the mixed non-sterile and sterile, soil treatments had a different pattern of infection, the percent root infection per pot of these treatments had a very narrow range 12.9% to 13.9%. It appears that mycorrhizae formation in the roots of *A. purpurea* may be under internal control whereby further root infection would be inhibited once the percent infection reaches an optimum level. This could be an advantage for the grass because further infection may not improve nutrient uptake above the cost of supporting the growth of the fungus. Research with ryegrass and sudan-grass showed that increased phosphate within the plant resulted in reduced percent mycorrhizae along with reduced soluble carbohydrates which may be a control mechanism (Menge et al. 1978; Jasper et al. 1979). Delaying initial mycorrhizal infection does not seem to enhance the growth of *A. purpurea* because when the upper layer of soil was sterile there was no significant difference from the non-sterile soil. However, if the upper layer of soil does not contain fungal propagules and exceeds 20 - 30 cm, the grass cannot produce enough dry mass to establish, grow, and survive, which has been reported for other species (Willis et al. 2013).

Soil generalist, such as many hymenopterans, may be important for the establishment and growth of *A. purpurea*. Species of ants like *S. invicta* the imported red fire ant have become dominant species in much of the southern United States. Despite claims of considerable environmental or ecosystem disservice, control of *S. invicta* seems to be lacking (Wills and Landis 2018). More attention should be paid to species like this, especially their belowground effects. Little is known about what to expect from introduced species in the future especially as the climate continues to change (Pagano and Gupta 2016; Wills and Landis 2018). Disturbance tolerant ants such as *S. invicta* are often

invasive and will most probably influence native species populations in a negative way (LeBrun et al. 2013; Moranz et al. 2013).

We examined the potential for *S. invicta* to be a vector of fungal propagules. *Solenopsis invicta* does not appear to act as a vector for the transport of fungal propagules which could establish mycorrhizal associations in the roots of *A. purpurea*. This is evidenced by the similar patterns of infection in the vertical rhizosphere of *A. purpurea* between treatments with and without ants. Perhaps *S. invicta* moving soil brought nutrients to the plant roots which in effect could replace the fungal hyphae (Wills and Landis 2018). Examination of the root dry mass allocation in the non-sterile and 20+/20- soil treatments, without *S. invicta* revealed that root dry mass increased primarily in the sterile soil regions of the pot. When *S. invicta* was present in the 20+/20- soil treatment, root dry mass appeared to increase only in the non-sterile soil regions but not in the sterile soil. However, in non-sterile soil only, root dry mass in each of the four 10 cm segments was higher in the presence of *S. invicta*. Also, the increase in *A. purpurea* total dry mass in the 20+/20- soil treatment with *S. invicta* compared to without the ant was almost one-half that seen in the non-sterile soil treatment.

The mechanism by which *S. invicta* could affect the growth of *A. purpurea* in non-sterile soil is not certain but seems likely to be at the level of the mycorrhizal symbiosis since changes in *A. purpurea* growth in the presence of *S. invicta* were greatest when roots were most highly infected and because *A. purpurea* is obligatorily dependent upon mycorrhizae for growth. However, looking at the results from across all experiments, the presence of mycorrhizal fungi in the roots of *A. purpurea* does not explain by itself the growth of the grass. When looking at *A. purpurea* plants that became infected with mycorrhizae across all experiments in time, there was not a significant linear correlation between percent infection and total *A. purpurea* dry mass (Pearson's correlation, Engelken 1995). This suggests that the relationship probably involves several factors that may not be easily identified or controlled. Plant growth and yield is optimized when nutrient uptake by the fungus and its delivery to the host plant is equivalent to, or exceeds, the carbohydrate demands of the fungus (Smith et al, 1994). It is possible that the presence of *S. invicta* may enhance the efficacy of the mycorrhizal symbiosis directly by reducing mycorrhizal fungal biomass through feeding on external hyphae (Lanza 1991). This reduction in fungal biomass may be sufficient to lower the requirements of the fungus imposed on the host without significantly diminishing nutrient uptake. Also, the venom of *S. invicta*, an alkaloid having antibacterial, antifungal and insecticidal activity could reduce other fungal biomass (Blum 1985). The dispersal of venom may also act indirectly by reducing the number of microorganisms in the soil (Bruno de Carvalho et al. 2019), which could act antagonistically toward the mycorrhizal symbiosis in a manner analogous to sterilization (Fitter and Garbaye 1994). Because significant effects on *A. purpurea* growth were not seen in all soil treatments, it is likely that other potential chemical, physical or biotic changes to the soil as a result of *S. invicta* activity could have been important factors influencing these findings.

Interpretation of these results and others are difficult due to the complexity of the interactions between *A. purpurea,* the mycorrhizal fungi, and the soil environment including *S. invicta*. Temporal effects such as timing of rainfall events or other environmental conditions could also be important when comparing experiments in time. Dry mass measures of *A. purpurae* were not noticeably changed over the two years of these experiments although percent infection did change (Engelken 1995). It seems that the effects of mycorrhizal symbiosis on the biomass of *A. purpurea* varies depending upon a number of factors that were not identified and appear difficult to control. The quantity of mycorrhizal infection in plant roots and the abundance of propagules in the soil can change throughout the season and timing of infection is likely to be important for plant growth (Abbott and Gazey 1994). Also, all mycorrhizal fungi do not contribute equally to nutrient uptake and plant growth and thus roots may be highly infected, but with a variety of fungi that have a wide range of effectiveness and responses to environmental conditions (Brundrett 1991; Wills and Landis 2018). Despite the difficulty in predicting the effects of unknown mixed factors on plant growth, it appears that *A. purpurea* is an obligate mycotroph dependent upon the fungi for its growth, but other soil factors including certain biota such

as *S. invicta* can potentially alter the effectiveness of the mycorrhizal symbiosis and consequently the growth of *A. purpurea*.

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