Characterizing the interaction between a fungal seed pathogen and a deleterious rhizobacterium for biological control of cheatgrass

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ABSTRACT

Two soil microorganisms have been shown to negatively affect the invasive species cheatgrass (Bromus tectorum) and have the potential to serve as biological control agents. The fungal pathogen Pyrenophora semeniperda kills mostly slow-germinating cheatgrass seeds and reduces the cheatgrass seedbank. It is naturally occurring within the western United States. The deleterious rhizobacterium Pseudomonas fluorescens D7 inhibits cheatgrass root elongation and seedling vigor. This paper characterized the interaction between the two microorganisms in order to determine if dual inoculations would provide more effective cheatgrass control. First, to determine the effect of P. fluorescens D7 on P. semeniperda activity, dormant cheatgrass seeds were exposed to the microorganisms in combination and alone. We found a slight increase in fungal infection with dual inoculations but a decrease in fungal-caused seed mortality. Second, to study the effect of P. semeniperda on P. fluorescens D7, non-dormant cheatgrass seeds were exposed to one or both microorganisms. We observed no increase in the inhibitory effect of P. fluorescens D7 with dual inoculations and in some cases saw less growth inhibition in the presence of both microorganisms. Overall, our findings suggest that there is no beneficial interaction between P. semeniperda and P. fluorescens D7 that provides improved cheatgrass control. Possible explanations include the production of antifungal metabolites by P. fluorescens D7, the production of antibiotics by P. semeniperda, or competition within the seed zone (i.e. spermosphere) for space or limited resources.

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1. Introduction

Cheatgrass (Bromus tectorum L.) is an invasive winter annual grass that has had a profound impact on semiarid plant communities in western North America. It was introduced to North America in the late 1800s (Mack, 1981) and since that time has acquired a widespread distribution, occurring in all 50 of the United States. Its impacts on native biodiversity include both direct and indirect negative effects. The rapid growth of cheatgrass in the spring allows it to out-compete many native species for resources and replace diverse native communities with monospecific cheatgrass stands (Booth et al., 2003; Humphrey and Schupp, 2004). Additionally, cheatgrass causes more frequent grass fires and alters nutrient dynamics, which negatively affect native species but benefit cheatgrass (Vasquez et al., 2008).

Traditional cheatgrass control methods are primarily focused on controlling above-ground biomass and include herbicides and tillage to reduce plant biomass (Peeper, 1984). These methods are limited in efficacy because they do not specifically target cheatgrass seeds within the seed bank and most are not species specific. Each year cheatgrass produces a large number of seeds, producing as many as tens of thousands of seeds per m² annually (Smith et al., 2008). A subset of these seeds may not germinate during their first year, but may carry over in the seed bank and germinate in the subsequent 2–3 years (Smith et al., 2008), making control methods that only target plant biomass less effective. An alternative control method may be the use of biological control agents that specifically target the seed stage and/or target cheatgrass specifically. Two soil microorganisms have been shown to negatively affect cheatgrass at the seed and/or seedling stage and have the potential to serve as biological pesticides.

Pyrenophora semeniperda (Brittlebank and Adam) Shoemaker (anamorph Drechslera campanulata (Lév.) B. Sutton) is a fungal pathogen that infects cheatgrass seeds in the soil and has been found to reduce the number of viable seeds in cheatgrass seed banks (Meyer et al., 2007). This fungus acts differently depending on the dormancy of seeds. Dormant seeds (i.e. with slow germination) suffer higher levels of P. semeniperda-caused mortality, while non-dormant seeds may be infected but often escape pathogen-caused mortality by germinating before the pathogen fully establishes (Beckstead et al., 2007). Temperature does influence P. semeniperda efficacy. It has an optimal temperature range from 15 °C to 25 °C (Campbell et al., 2003). Given this information,
P. semeniperda is likely active from late spring to fall in western North America but inactive during the winter months. Pyrenophora semeniperda is naturally occurring in soils throughout the western United States from a variety of habitat types (i.e. semiarid shrub-steppe to Palouse prairie grassland; Meyer et al., 2007). Because P. semeniperda can target the cheatgrass seed bank, it shows promise as a biological control agent. It produces macroscopic, black stromata that protrude from the seed, facilitating detection of diseased seeds in the seed bank.

Pseudomonas fluorescens Migula strain D7 is a deleterious bacterium that inhibits cheatgrass root elongation and lowers overall plant vigor (Kennedy et al., 1991). It was originally isolated from a winter wheat root in eastern Washington (1991). This rhizobacterium colonizes cheatgrass roots and produces a phytotoxin that inhibits cheatgrass (Tranel et al., 1993). However, application of P. fluorescens D7 has not been shown to kill cheatgrass seeds in prior laboratory and field experiments (Kennedy et al., 1991). It is fairly specific to cheatgrass and does not have widespread effects on non-target species (Kennedy et al., 2001). Temperature also affects P. fluorescens D7 activity. It operates at cooler temperatures than P. semeniperda, with an ideal range from 15 °C to 20 °C (A. Kennedy, personal communication). Thus, P. fluorescens D7 is active in western North America during the late fall and early spring. Because P. fluorescens D7 can reduce cheatgrass biomass, it also has the potential to serve as a biological control agent.

The order in which cheatgrass seeds naturally encounter these organisms is likely to vary by season. In the fall cheatgrass seeds will germinate quickly following a rain event. Pyrenophora semeniperda will be active throughout the fall and as temperatures drop P. fluorescens D7 activity will begin. We would expect that cheatgrass seeds could potentially be exposed to P. fluorescens first and later P. fluorescens D7 as temperatures drop or that seeds may be exposed to both microorganisms at the same time. During the fall we would also expect low levels of fungal-caused mortality because seeds germinate quickly. Conversely, in the spring cheatgrass seeds will germinate slowly. Pseudomonas fluorescens D7 will be active throughout the spring and as temperatures increase P. semeniperda will also become active. Thus, cheatgrass seeds could potentially be exposed to P. fluorescens D7 first followed by P. semeniperda after temperatures increase. Seeds also may be exposed to both microorganisms simultaneously. During the spring we expect high levels of fungal-caused mortality because seeds germinate more slowly.

Although both of these organisms hold promise as biological control agents, it is unlikely that they can completely control cheatgrass when applied individually. They are limited in their efficacy because P. fluorescens D7 has not been shown to kill cheatgrass seeds and P. semeniperda usually does not kill seeds that germinate quickly. However, it is possible that applying these organisms in combination will allow for more effective cheatgrass control. The idea of combining biological control agents has received increasing attention in recent years as a possible way of improving success and reducing variability in biological control efforts (Chandramohan and Charudattan, 2003; Denoth et al., 2002; Guetsky et al., 2001). In a review of biological control programs that introduced multiple agents, Denoth et al. (2002) report that the success of weed control programs increased with the number of biological control agents released. However, the programs reviewed were limited to those that released insect pests or fungi. Although biological control agents may positively interact, they can also negatively impact each other or have no effect (Whipps, 2001).

Given previous experiments combining bacteria and fungi it is hypothesized that there will be a positive interaction between P. semeniperda and P. fluorescens D7. Two predictions extending from this hypothesis are (1) that the presence of P. fluorescens D7 will increase the level of P. semeniperda-caused mortality in dormant cheatgrass seeds and (2) that P. semeniperda will enhance the inhibitory effect of P. fluorescens D7. The results of this study have implications for cheatgrass control and restoration projects that depend on more effective control of cheatgrass. Additionally, they will help to illuminate the previously unexplored area of the interaction between microbial agents for biological control of invasive plants.

2. Materials and methods

2.1. Preparation of inoculum in culture

Pseudomonas fluorescens D7 stocks were obtained from Ann Kennedy of USDA-ARS, Pullman, WA, and stored at −80 °C in 6.8 M glycerol. Frozen cultures were plated on Sands and Rovira (1970) agar and grown for 48 h at 20 °C. After 48 h, the culture was transferred to PMS (Pseudomonas minimal salts, Bolton et al., 1989) liquid medium. Cultures were grown at 20 °C shaking at 200 rpm, for 48 h, reaching an estimated density of 10^8 cfu/ml.

To prepare the fungal inoculum, conidia were produced from a P. semeniperda isolate from Whitertocks, Tooele County, UT. Conidial inoculum was obtained by first culturing surface-sterilized stromata from killed seeds found in cheatgrass seed bank samples from the site on V8 agar (Beckstead et al., 2007), then transferring stromata produced in V8 agar culture to MAM (modified alphacell medium) agar culture, which promotes direct production of conidia on the mycelial surface (Campbell et al., 1996). These were harvested by rinsing the plates with sterile water, filtering the conidial suspension on a 25-μm sieve, and air-drying the filtrate.

Cheatgrass seeds were collected in July 2008 from a monoculture of cheatgrass located in Spanish Fork, Utah County, UT. Half of the seed collection was stored at room temperature to permit seeds to lose dormancy and half was stored at −18 °C to maintain seeds in a state of primary dormancy (Beckstead et al., 2007). Prior to use in experiments, seeds were surface sterilized by soaking for 1 min each in 70% ethanol, 10% bleach, and DI water, and then allowed to air dry.
2.2. Experiment one: does bacterial P. fluorescens D7 enhance the activity of fungal P. semeniperda at the seed stage?

The purpose of this experiment was to investigate the interaction between P. fluorescens D7 and P. semeniperda in the spring in order to determine if the presence of P. fluorescens D7 increases fungal-caused death and fungal infection. We specifically chose the spring season because P. semeniperda activity is the most ecologically significant in the spring when seeds germinate slowly; prior data found the fraction of field-killed seeds by P. semeniperda was greatest in the spring across 5 years at two contrasting sites (S. Meyer, personal communication). In our experiment there were four controls and three experimental treatments. The controls were a water control, a PMS media control for the bacteria, a P. semeniperda control and a P. fluorescens D7 control. The fungal media control, agsorb, was not included. Preliminary data found that agsorb media did not have a significant effect on fungal conidial germination (Agsorb treatment: average 75% conidial germination, n = 100; Control treatment: average 96% conidial germination, n = 100; Contingency Likelihood analysis, Chi-square value 1.71, P = 0.1914) or B. tectorum seed germination at 14 days (Agsorb treatment: average 78% germination ± 4% SD; Control treatment: average 87% germination ± 16% SD; t-test analysis, t-value 1.35, P = 0.2234). The experimental treatments were (1) exposure to both microorganisms at planting, (2) exposure to P. semeniperda at planting and P. fluorescens D7 2 days after planting, and (3) exposure to P. fluorescens D7 at planting and P. semeniperda 4 days following planting.

The experiment was carried out in Petri dishes (100 x 15 mm) using sterile sand as a growth medium. Twelve dormant cheatgrass seeds were placed around the perimeter of the dish and treatments were applied as described above. To inoculate with P. semeniperda, 10 mg of dry conidia were diluted in 300 mg of sterile Agsorb (clay carrier: Agsorb Products, Chicago, IL, USA). This medium was then evenly spread over the Petri dish. To inoculate seeds with P. fluorescens D7, 2 ml of culture was evenly pipetted over the Petri dish. PMS media was applied in an identical manner in the PMS control, as was water in the water control. All dishes were incubated at 15 °C without lights and maintained at 10% moisture by daily applications of DI water following planting.

To determine the effect of combining both microorganisms on the activity of P. semeniperda, fungal infection and fungal-caused mortality were measured in each dish by examining seeds for P. semeniperda stromata after 5 weeks. Fungal infection was calculated as the sum of seeds killed by the fungus (ungerminated seeds with fungal stroma) and seeds that were infected but still germinated (germinated seeds with fungal stroma).

This experiment contained seven inoculation treatments with 20 replications each in a sand medium. Each replicate consisted of a Petri dish with 12 seeds. The experiment was repeated in water agar medium; however, the analysis in this paper focuses on the data from the sand medium. The effect of inoculation treatments on average root length and mean seed germination were analyzed using analysis of variance (ANOVA; JMP from SAS 2007). The response variables were transformed to improved homogeneity of variance prior to analysis (root length, square-root and proportion germinated, arc sine square-root). The Tukey–Kramer HSD multiple comparison test (P < 0.05) was used for means separations.

2.4. Experiment three: does fungal P. semeniperda enhance the inhibitory effect of bacterial P. fluorescens D7 at the seedling stage?

To supplement data from Experiment Two, the effect of P. semeniperda on P. fluorescens D7 inhibition during the fall was also investigated at the seedling stage. There were four controls and one experimental treatment. The four controls were as follows: a water control, a PMS media control, a P. semeniperda control and a P. fluorescens D7 control. In the P. semeniperda control seeds were inoculated with P. semeniperda conidia before planting. For the P. fluorescens D7 control seeds were inoculated with bacteria 48 h after planting. For the experimental treatment seeds were inoculated with P. semeniperda before planting and then inoculated with P. fluorescens D7 48 h after planting. There were two harvest dates (at 5 and 8 weeks after planting) and 28 replications per treatment per harvest date. This experiment was repeated with a modification in the timing of inoculations at 2 weeks. The results from this 2-week inoculation experiment found no treatment effects for P. fluorescens D7 (at both harvest dates the P. fluorescens D7 control did not differ significantly from the media control in above- and below-ground biomass, P < 0.05), indicating that P. fluorescens D7 must colonize the roots of young seedlings to be effective.

Three non-dormant cheatgrass seeds (thinned to one at 1 week) were planted in each of the 56 pots (2.1 x 21 cm UV stabilized Cone-tainers (Stuewe and Sons Inc., Corvallis, OR) filled with a sterile 2:1:1 sandy loam, peat moss and turf mix (Wittkopf Landscaping, Spokane, WA)) for each of the five treatments for a total of 280 pots and 840 seeds. For the water control, PMS control, and P. fluorescens D7 control non-inoculated cheatgrass seeds were planted. For the P. semeniperda control and the experimental treatment, cheatgrass seeds that had been inoculated with P. semeniperda were planted. To inoculate seeds, 0.0025 g dry conidia and 50 cheatgrass seeds were placed in a 4 ml glass vial and vi-
brated for 1 min with a modified sander to ensure even coverage of conidia on seeds. At 48 h after planting, the *P. fluorescens* D7 control and experimental treatment were inoculated with *P. fluorescens* D7. To inoculate soil with *P. fluorescens* D7, 3 ml of culture was pipetted directly onto the soil. At this time the PMS and water control treatments were inoculated with PMS media or water in an identical manner. All plants were grown in Conviron environmental growth chambers with 12 h of light per day and a temperature cycle from 15 °C and 25 °C (12 h each). Soil moisture was maintained between 5% and 15% by watering with DI water four times per week and plants received a 25% solution of Peter’s all-purpose plant food every 2 weeks. All plants were randomized and rotated weekly.

At 5 and 8 weeks plants were harvested to determine the effect of *P. semeniperda* on *P. fluorescens* D7 activity. Plants were rinsed to remove soil particles and separated into aboveground and belowground parts. Both the roots and shoots were dried at 60 °C for 72 h and then weighed to obtain dry biomass.

This experiment contained five inoculation treatments with 28 replications each with inoculation with *P. fluorescens* D7 at 48 h. The experiment was repeated in time with a harvest at 5 and 8 weeks. Each replicate consisted of a seedling grown in a cone-tainer. The entire replicated experiment was repeated with a modification in the timing of inoculations at 2 weeks as stated above; however the analysis in this paper focuses on the data from the 48-h inoculation. Data were transformed with a square-root transformation and analyzed using analysis of variance (ANOVA; JMP SAS 2007). For each harvest date, treatment served as the independent variable and dry root biomass or dry shoot biomass were the response variables. To compare the different soil treatments within each harvest date, we used the Tukey–Kramer HSD test for multiple comparisons (sample sizes: W-control, M-control, F-only, B-only, and F and B at 5 wks were 27, 28, 20, 28, and 21, respectively, and at 8 wks were 28, 28, 25, 28, and 24, respectively). The variation in sample size was due to variability in seed germination.

### 3. Results

#### 3.1. The effect of bacterial *P. fluorescens* D7 on fungal *P. semeniperda* activity

We initially predicted that dormant cheatgrass seeds grown in the presence of *P. fluorescens* D7 and *P. semeniperda* would have higher levels of *P. semeniperda*-caused infection and mortality. Fungal-caused infection and death differed significantly between treatments (DF = 6, F = 2167.36, P < 0.0001 and DF = 6, F = 289.36, P < 0.0001 for infection and death, respectively; Fig. 1). Treatments that were not exposed to *P. semeniperda* did not show any significant level of fungal-caused death or infection, indicating that there were no background levels of infection in the seed population used. There were high levels of fungal infection (>0.95%) in all treatments exposed to the fungus, and most of the infected seeds were also killed by the fungus. The numbers of infected seeds in the three treatments containing both microorganisms did not differ significantly from each other but were slightly, although significantly, higher (an increase of 5%) than the number of infected seeds in the fungus-only control (P < 0.05; Fig. 1). Additionally, the proportion of killed seeds in the experimental treatments containing both organisms did not differ significantly from the fungus-only control. However, when the bacterium was applied before the fungus there was on average a 16% reduction in the proportion of killed seeds in comparison to the other treatments containing both microorganisms (Fig. 1).

#### 3.2. The effect of fungal *P. semeniperda* on bacterial *P. fluorescens* D7 activity: germination stage

We further predicted that the presence of the fungus would enhance the inhibitory effect of the bacterium, leading to a lower proportion of germinated seeds and shorter root lengths in non-dormant cheatgrass seeds exposed to both microorganisms. At the germination stage, the average root length of germinated seeds differed significantly among treatments (DF = 6; F = 40.72; P < 0.0001; Fig. 2). Seeds in the water control had the longest root lengths and root lengths decreased with exposure to one or both of the microorganisms. The fungus-only control had 34% shorter root lengths than the water control. Exposure to *P. fluorescens* D7 in isolation decreased root lengths by 44% in comparison to the media control. The effect of *P. semeniperda* on *P. fluorescens* D7 varied with the order that seeds were exposed to the microorganisms. When both microorganisms were applied at the same time or when the bacterium was applied first there was no significant difference in root length compared to the bacteria-only control (P < 0.05; Fig. 2). However, in comparison to the bacteria-only control, root length increased 36% when the fungus was applied before the bacterium (P < 0.05; Fig. 2).

Exposure to one or both microorganisms also significantly decreased the proportion of seeds that germinated (DF = 6; F = 35.00; P < 0.0001; Fig. 2), with the fungus-only control having the lowest germination. Both the water and media controls showed high (>95%) germination, indicating that in the absence of the microorganisms seeds were highly viable. Germination was decreased by 55% in the fungus-only control and 18% in the bacteria-only control in comparison to the water and media controls, respectively. When *P. semeniperda* was applied simultaneously with *P. fluorescens* D7 or when *P. fluorescens* D7 inoculation occurred 2 days following *P. semeniperda* inoculation there was no significant reduction in germination as compared to the bacteria-only control (P < 0.05; Fig. 2). When *P. fluorescens* D7 was applied prior to the fungus, 22% fewer seeds germinated in comparison to the bacteria-only control (Fig. 2). It should be noted...
that the bacterium lowered the ability of the fungus to reduce germination, as evidenced by the increase in germination with dual inoculations in comparison to the fungus-only control.

3.3. The effect of fungal P. semeniperda on bacterial P. fluorescens D7 activity: seedling stage

We also tested the prediction that the fungus would increase the inhibitory effect of the bacterium at the seedling stage. Given this prediction we would expect to see lower root and shoot biomass in the treatment containing both microorganisms. Above-ground biomass at 5 weeks differed among treatments (DF = 4; \( F = 12.23; P < 0.0001 \); Fig. 3). The media control had significantly higher shoot biomass than all other treatments and the water control had the lowest. Relative to the water control, the fungus-only treatment showed an increase of 47% in shoot biomass. In comparison to the media control, the bacteria-only control showed a significant reduction in root biomass (\( P < 0.05 \); Fig. 3). The treatment containing both microorganisms did not differ significantly from treatments containing only the bacterium or only the fungus. The pattern in shoot mass at 8 weeks varied slightly from the 5-week data. The experimental treatment showed a 34% increase in shoot biomass compared to the fungus-only control but was not significantly different from the bacteria-only control (\( P < 0.05 \); Fig. 3).

At 5 weeks after planting, root biomass was significantly higher in the media control than in any other treatment (DF = 4; \( F = 9.79; P < 0.0001 \), increasing 35% compared to the water control (Fig. 3). All other treatments did not differ significantly from each other. At 8 weeks after planting root biomass exhibited larger differences. The media control, the bacteria-only control, and the experimental treatment were significantly higher than the remaining treatments but did not differ from each other (\( P < 0.05 \)). At 8 weeks there is a clear fertility effect of the medium, demonstrated by the significantly higher root biomass in all treatments containing medium.

4. Discussion

This research evaluated the interaction between a fungal and a bacterial biological control agent of cheatgrass to determine if dual inoculation by both organisms provided enhanced cheatgrass control. We initially expected a positive relationship between the two
microorganisms. More specifically, we predicted higher levels of fungal-caused infection and mortality of seeds in the presence of \( P. \) \textit{fluorescens} D7 (Experiment One) and an increased level of plant growth inhibition in the presence of \( P. \) \textit{fluorescens} D7 and \( P. \) \textit{semeniperda} (Experiments Two and Three). In general, our results do not indicate that there is a positive interaction between the two microorganisms that leads to more effective cheatgrass control.

4.1. The effect of bacterial \( P. \) \textit{fluorescens} D7 on fungal \( P. \) \textit{semeniperda}

We found an increase, although small, in fungal infection when both organisms were applied in combination. However, when \( P. \) \textit{fluorescens} D7 was applied prior to \( P. \) \textit{semeniperda} there was a reduction in fungal-caused mortality (Fig. 1). These results do not indicate a clear positive or negative relationship between the two biological control agents but at least suggest that dual inoculation does not improve cheatgrass control. There are several potential reasons for the absence of a positive effect of \( P. \) \textit{fluorescens} D7 on \( P. \) \textit{semeniperda}. \textit{Pseudomonas} species have been shown to produce antifungal metabolites that inhibit the activity of fungi (Nielsen et al., 1998). In a study on metabolites produced by \( P. \) \textit{fluorescens} D7, Gurusiddaiah et al. (1994) reported that an active compound produced by the bacterium was inhibitory to the plant pathogenic fungus \textit{Gaeumannomyces graminis}. Thus, it is plausible in this set of experiments that \( P. \) \textit{fluorescens} D7 also exhibited some degree of antifungal activity. To date no microbial-focused studies exist that directly document antifungal activity specifically towards \textit{Pyrenophora} species. However, it is important to note that fluorescent pseudomonads have been found to interact beneficially with some fungi species to provide more effective biological control (DuJiff et al., 1999; Duffy et al., 1996). Future studies should directly examine how active compounds produced by \( P. \) \textit{fluorescens} D7 directly affect \( P. \) \textit{semeniperda} in order to complement the data presented here.

Alternatively, the absence of a positive effect of \( P. \) \textit{fluorescens} D7 on \( P. \) \textit{semeniperda} activity may be the result of a competitive interaction between the two microorganisms. Previous studies have shown that bacteria and fungi compete for iron (Larkin and Fravel, 1998; O’Sullivan and O’Gara, 1992) and specifically that \textit{Pseudomonas} species may be involved in this type of competitive interaction (DuJiff et al., 1993). Given that this experiment was carried out in the seed zone (i.e. spermosphere). Nelson (2004) notes that the seed zone is a highly competitive atmosphere in which rapid occupation is critical for microorganism establishment and growth. Clearly, the presence of \( P. \) \textit{fluorescens} D7 did not eliminate fungal activity. However, when seeds were exposed to \( P. \) \textit{fluorescens} D7 prior to \( P. \) \textit{semeniperda} there was a reduction in fungal-caused seed mortality. This finding supports the idea that \( P. \) \textit{fluorescens} D7 and \( P. \) \textit{semeniperda} compete for a resource or location and the organism that is applied first has the advantage.

4.2. The effect of fungal \( P. \) \textit{semeniperda} on bacterial \( P. \) \textit{fluorescens} D7: germination stage

Our data provides no conclusive evidence for a positive effect of \( P. \) \textit{semeniperda} on \( P. \) \textit{fluorescens} D7 at the germination stage. Root length decreased with single- and dual-organism inoculations. However, when the fungus was applied before the bacteria root lengths were significantly longer than in the bacteria-only control (Fig. 2a). This finding also supports the idea that the organism that is applied first has an advantage in colonizing the seed zone and/or utilizing limited resources (i.e. the competitive interaction hypothesis outlined above). Alternatively, the relationship between \( P. \) \textit{semeniperda} and \( P. \) \textit{fluorescens} D7 may be one of antibiosis. Antibi-

otic production by fungi has long been demonstrated in fungi that are used as biological control agents of plant pathogens (Di Pietro, 1995; Howell, 1998). The most common isolates found to produce antibiotics are \textit{Trichoderma/Gliocladium} species (1998), but it is also possible that \( P. \) \textit{semeniperda} may produce antibiotics. Whipp (2001) notes that antibiotic production by fungi is poorly understood in comparison to antibiotic production by biocontrol bacteria. Thus, the antibiotic production capabilities of \( P. \) \textit{semeniperda} should be explored in more depth to complement the findings presented here.

For the most part, the germination data also show no positive relationship between \( P. \) \textit{semeniperda} and \( P. \) \textit{fluorescens} D7. Germination decreased with exposure to one or both microorganisms. Two of the three dual-organism inoculations did not differ from the bacteria-only control but when the bacterium was applied before the fungus significantly fewer seeds germinated (Fig. 2b). However, this finding should not be taken as evidence for a positive interaction between \( P. \) \textit{semeniperda} and \( P. \) \textit{fluorescens} D7. Rather, it demonstrates that the presence of the bacterium lowers the ability of the fungus to inhibit germination in non-dormant seeds. Thus, these data further support the findings from Experiment One in which the bacteria limited fungal-caused seed mortality.

4.3. The effect of fungal \( P. \) \textit{semeniperda} on bacterial \( P. \) \textit{fluorescens} D7: seedling stage

Plant biomass data at both 5 and 8 weeks failed to demonstrate a positive effect of \( P. \) \textit{semeniperda} on the inhibitory activity of \( P. \) \textit{fluorescens} D7. At both harvest dates, the dual inoculation treatment did not significantly differ from the bacteria-only control (Fig. 3). These findings can likely be explained by the competitive interaction or antibiosis hypothesis outlined above. It is further important to note that although we saw a reduction in root length in young seedlings (1 week old; Experiment Two) exposed to \( P. \) \textit{fluorescens} D7, we did not observe a consistent reduction in plant biomass as has been seen in previous studies. The harvest dates for the seedling experiment occurred 5 and 8 weeks following planting. It is unclear how long \( P. \) \textit{fluorescens} D7 stays active and present in the soil and it is possible that both of these harvest dates were outside of the window of time that \( P. \) \textit{fluorescens} D7 remains highly active within soil. Future experiments should examine the effect of \( P. \) \textit{fluorescens} D7 and \( P. \) \textit{semeniperda} on plant biomass at shorter intervals of time.

5. Conclusions

Despite some irregularities, our results overall indicate that \( P. \) \textit{semeniperda} and \( P. \) \textit{fluorescens} D7 do not interact positively and may even interact competitively. Given this characterization, it is unlikely that dual applications of these microorganisms will lead to more effective cheatgrass control. However, microorganism combinations may still be helpful for improving the consistency of cheatgrass biological control efforts or for providing control in a wider array of environmental conditions. Additional experiments should examine how the interaction between these organisms shifts with altered growth conditions (e.g. temperature, soil moisture, nutrient availability). Furthermore, other soil biota are known to be important in determining the interaction between biological control agents (Whipp, 2001). Although \( P. \) \textit{fluorescens} D7 and \( P. \) \textit{semeniperda} may not interact positively to provide control, both organisms still have the potential to be powerful biological control agents when applied on their own or when used in combination with traditional control methods.
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