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Research Article

Survival of a Rifampicin-Resistant *Pseudomonas fluorescens* Strain in Nine Mollisols

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Pseudomonas fluorescens strain D7 (*P.f.* D7) is a naturally occurring soil bacterium that shows promise as a biological herbicide to inhibit growth of annual grass weeds, including downy brome (*Bromus tectorum* L.), in crop- and rangelands. *Pseudomonas fluorescens* strain D7rif (*P.f.* D7rif) is a rifampicin-resistant strain of *P.f.* D7. One of the greatest obstacles to successful biological weed control is survival of the organism under field conditions. Nine soils in the taxonomic order of Mollisols, collected from downy brome-infested areas of the Western and Central United States, were inoculated with *P.f.* D7rif and incubated in the laboratory to determine the effects of soil type, soil properties, incubation temperature, and soil water potential on survival of *P.f.* D7rif over 63 days. Silt loam soils from Lind, Washington, and Moro, Oregon, sustained the highest *P.f.* D7rif populations, and recovery was the lowest from Pendleton, Oregon soil. Survival and recovery of *P.f.* D7rif varied with soil type and temperature but not with the two soil water potentials tested. After 63 days, *P.f.* D7rif was recovered at levels greater than log 5.5 colony forming units (CFU) g^{-1} soil from five of the nine test soils, a level adequate to suppress downy brome under field or range conditions.

1. Introduction

The annual grass weed downy brome, or cheatgrass (*Bromus tectorum* L.), infests 22 million ha in the Western United States [1] and has degraded croplands used for small grain production and rangelands used for grazing. In Pacific Northwest croplands, severe infestations of downy brome may reduce wheat (*Triticum aestivum* L.) yield by an average of 27% [2] depending on location, plant density, and time of weed emergence [3, 4]. Because it has a similar life cycle to wheat, downy brome is extremely competitive with wheat for scarce moisture and nutrients. Tillage, residue burning, and herbicides are the control options available to wheat producers; however, each of these options may be undesirable for sustainable crop production. Tillage may leave soil vulnerable to erosion, and burning of crop residues is detrimental to air quality. Herbicides to control downy brome in wheat are expensive and chemical residue may limit crop rotation options. In rangelands, downy brome continues to overrun grazing areas, mainly due to overgrazing and disturbance by humans. In Idaho and Utah, 5 million ha of rangeland is monoculture downy brome due to overgrazing [2]. Downy brome may provide some value for grazing early in the growing season; however, it dries quickly in the summer, poses a serious hazard for wildfires, and increases the frequency of fires leading to increased erosion and sedimentation [1]. As in cropland, options for controlling downy brome in rangelands are limited.

Pseudomonas fluorescens strain D7 (*P.f.* D7) is a naturally occurring soil bacterium isolated from wheat roots that has shown promise for selectively controlling downy brome in cropland [5]. When applied in the fall, *P.f.* D7 colonizes the roots of downy brome, inhibiting its growth and allowing desirable species to gain a competitive advantage. The bacteria require cool, moist conditions in order to survive at levels high enough to colonize roots and inhibit downy brome growth; however, weather conditions in the interior Western USA are often warm and dry well into the fall months. As part of ongoing research to determine the most optimum soil conditions for survival and efficacy of *P.f.* D7, a study was

Table 1: Chemical properties of test soils used in *P. fluorescens* D7rif survival study.

Location	pH	P ug g^{-1}	K ug g^{-1}	Organic matter %	NO_3-N ug g^{-1}	NH_4-N ug g^{-1}	Cation exchange capacity meq 100 g^{-1}
Akron, CO	6.0	268	536	1.17	56.7	3.01	18.3
Hays, KS-“A”	7.58	19.5	316	1.14	60	2.1	15
Hays, KS-“B”	5.49	8.7	552	2.3	58	2.57	21.6
La Crosse, WA	5.75	6.1	456	2.22	5.6	5.5	14.6
Lewiston, ID	5.29	9.3	680	5	17.3	16.6	24.5
Lind, WA	6.38	9.3	472	1.05	4.8	4.78	14.3
Moro, OR	5.69	12.9	472	1.52	14.8	3.05	13.4
Pendleton, OR	5.18	6.8	568	2.43	63.3	3.83	19.4
Pullman, WA	5.3	15.2	460	4.43	9.3	NA	18.3

Table 2: Particle size distribution and USDA soil series, textural classes, and taxonomic classification of soils used in *P. fluorescens* D7rif survival study.

Location	Particle size distribution (%)			USDA soil series/textural class/taxonomic classification [9][#]
	Sand	Clay	Silt	
Akron, CO	40	12	48	Platner loam; fine, smectitic, mesic Aridic Paleustolls
Hays, KS-“A”	25.6	15.2	59.2	Roxbury silt loam; fine-silty, mixed, superactive, mesic Cumulic Haplustolls
Hays, KS-“B”	21.6	23.2	55.2	Crete silty clay loam; fine, smectitic, mesic pachic Udertic Argiustolls
La Crosse, WA[*]	36	10	54	Walla Walla silt loam; coarse-silty, mixed, superactive, mesic Typic Haploxerolls
Lewiston, ID	21.6	19.2	59.2	Broadax silt loam; fine-silty, mixed, superactive, mesic Calcic Argixerolls
Lind, WA	38	8	54	Ritzville silt loam; coarse-silty, mixed, superactive, mesic Calcidic Haploxerolls
Moro, OR[*]	33.6	11.2	55.2	Walla Walla silt loam; coarse-silty, mixed, superactive, mesic Typic Haploxerolls
Pendleton, OR[*]	29.6	15.2	55.2	Walla Walla silt loam; coarse-silty, mixed, superactive, mesic Typic Haploxerolls
Pullman, WA	26.4	14.4	59.2	Palouse silt loam; fine-silty, mixed, superactive, mesic pachic Ultic Haploxerolls

[#] All soils are of the order Mollisols.
[*] Walla Walla silt loam soil was collected at three locations.

conducted using nine soils collected from areas of the United States that are plagued by severe downy brome infestations. The objective of this study was to determine the effect of soil type, soil properties, incubation temperature, and soil moisture on survival of a rifampicin-resistant strain of the potential biological control agent *Pseudomonas fluorescens* D7 (*P.f.* D7rif). In a second study, we examined survival of *P.f.* D7rif applied to peat granules to determine whether survival of the bacteria could be enhanced by these materials. We hypothesize that application of the bacteria to peat granules might encapsulate and protect *P.f.* D7 to improve survival and efficacy under field conditions.

2. Materials and Methods

2.1. Soil Sample Collection. Soil samples were collected from the surface 15 cm at nine locations in the Western and Central United States where downy brome had been responsible for severe yield losses in croplands (Tables 1 and 2). Soils were chosen to represent the major soil types within the range of organic matter levels of the Northwest and North Central United States with downy brome infestations. The soils were collected from three sites in Washington state (Lind, La Crosse, and Pullman), two sites in Oregon (Moro and Pendleton), one site in Idaho (Lewiston), one site in Colorado (Akron), and two sites in Kansas (both near Hays; designated as “A” and “B”). At each site, soil was collected from the top four inches of seven random areas in each agricultural field. The soils were kept cool until used in the incubation studies, which occurred within two weeks. Prior to these incubation studies, visible organic material was removed from soils by hand, and soils were sieved to pass a 2 mm screen. Soil moisture curves were constructed for each of the soil types using the pressure plate method [6], and soil characteristics (pH, P, K, organic matter, NO_3-N, NH_4-N, and

Table 3: ANOVA analysis of *P. fluorescens* D7rif survival by soil type, incubation temperature, and soil moisture over the 63-day incubation study ($P < 0.05$).

Source	df	Day 3	Day 7	Day 10	Day 14	Day 21	Day 28	Day 63
Soil	8	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Temperature	2	0.0255	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Moisture	1	0.1162	0.0252	0.1071	0.0777	0.1983	0.4516	0.7718
Soil ∗ temperature	16	0.043	0.1642	0.1715	0.2216	0.686	0.2825	0.1815
Soil ∗ moisture	8	0.6808	0.5345	0.7848	0.767	0.8757	0.951	0.9972

cation exchange capacity) were determined by the University of Idaho soil analysis lab. Properties for each of the nine soils are listed in Table 1, and soil particle size distribution, USDA textural class, and taxonomic classification are listed in Table 2. Using the moisture curves constructed for each soil, the percent gravimetric soil moisture was calculated to bring each soil to 0.03 MPa and 0.10 MPa. The appropriate amount of deionized water was added to each soil, and soils were thoroughly mixed.

2.2. Bacterial Cultures. *Pseudomonas fluorescens* strain D7rif was selected from a population of *P.f.* D7 colonies that were resistant to 100 mg L^{-1} rifampicin when grown on Sand's agar [7] amended with rifampicin ($Sands_{rif}$). The *P.f.* D7rif stock cultures to be used in these experiments were maintained in 50% glycerol at −80°C. Liquid cultures of *P.f.* D7rif, grown in *Pseudomonas* minimal salts (PMS) broth [8] and prepared using an orbital shaker, were made 48 hours prior to inoculating soils. *P.f.* D7rif cultures were serially diluted and plated to determine initial log colony forming units (CFU) mL^{-1} inoculum prior to inoculating soils. Initial concentration of *P.f.* D7rif was log 9.0 CFU mL^{-1} for the incubation study and log 10.2 CFU mL^{-1} for the peat granule study.

2.3. Incubation Study. After adding the appropriate amount of moisture to each soil type, soils were spread onto a tray and 10 mL *P.f.* D7rif inoculum was applied to 100 g d.w. of soil using an atomizer. Soils were mixed thoroughly and placed in resealable plastic bags. Soils of each type and each of the two moisture contents (0.03 and 0.10 MPa) were incubated at three temperatures: 5, 10, and 20°C. There were two replications for each soil type-temperature-moisture combination, plus a nontreated control for each soil type at both moisture levels that was incubated at 10°C. Prior to addition of the *P.f.* D7rif inoculum, 2 g of each soil was serially diluted, and soil dilutions were plated onto MMN, tryptic soy, potato dextrose, proteolytic, Sands, and $Sands_{rif}$ agar in order to determine background numbers of rifampicin-resistant organisms as well as total pseudomonads (data not shown). No rifampicin-resistant organisms were detected in any of the soils prior to inoculation.

Soils were sampled at the time of application of *P.f.* D7rif and then again 3, 7, 10, 14, 21, 28, and 63 days after inoculation. At each sampling time, soils were mixed, bags were opened, and soils were quickly sampled. Bags were closed quickly to avoid moisture loss and contamination. Three replicate samples were removed from each bag at each sampling time. Soils were serially diluted, and the appropriate dilutions were plated onto Sands, $Sands_{rif}$, and tryptic soy agar. Only the results of the counts from $Sands_{rif}$ agar will be reported here. Dilution plates were incubated at 22°C, and colonies were counted after 48 hours of growth. The entire experiment was performed twice. The relationships between both experiments were similar, and so only results from the second experiment are reported. There were no rifampicin-resistant bacteria detected in the control samples for any of the soil types at any sampling time.

2.4. Survival of P.f. D7rif in Peat Granules. Peat granules were inoculated with *P.f.* D7rif to determine whether survival might be improved over simply inoculating directly onto the soil. The initial pH of the peat granules was 4.3, which is considered too low for optimum survival of *P.f.* D7rif. Peat granules were prepared for the study by autoclaving three times for 15 minutes each and amending with calcium carbonate (10% of peat granules dry weight) for a final pH of 6.0. Before inoculating with *P.f.* D7rif, serial dilutions of peat granules were plated onto $Sands_{rif}$ media, and no rifampicin-resistant bacteria were detected. *P.f.* D7rif cultures were prepared as described above and 50 mL of culture was added to the peat granules in resealable plastic bags. Peat granules and bacteria were mixed thoroughly. Moisture content of the peat granules for the duration of the study was 25%, and incubation temperature was 20°C. The mixture of peat granules and *P.f.* D7rif was sampled aseptically at the time of inoculation, and at days 2, 7, 9, 16, 23, 30, and 37, with 2 g material removed for serial dilutions and plating onto $Sands_{rif}$ agar as described above.

2.5. Statistical Analysis. Differences in survival of *P.f.* D7rif among soil types, temperatures, and soil moisture contents, as well as variances in the peat granule study, were determined by ANOVA analysis with JMP software using Tukey-Kramer HSD at the 0.05 level [10]. Correlation of survival with soil characteristics was determined through simple regression analysis [11].

3. Results

3.1. Incubation Study: Survival and Recovery of P.f. D7rif. Results of ANOVA analysis for *P.f.* D7rif recovery and survival over the 63-day incubation period are shown in Table 3. *Pseudomonas fluorescens* D7rif recovery was affected

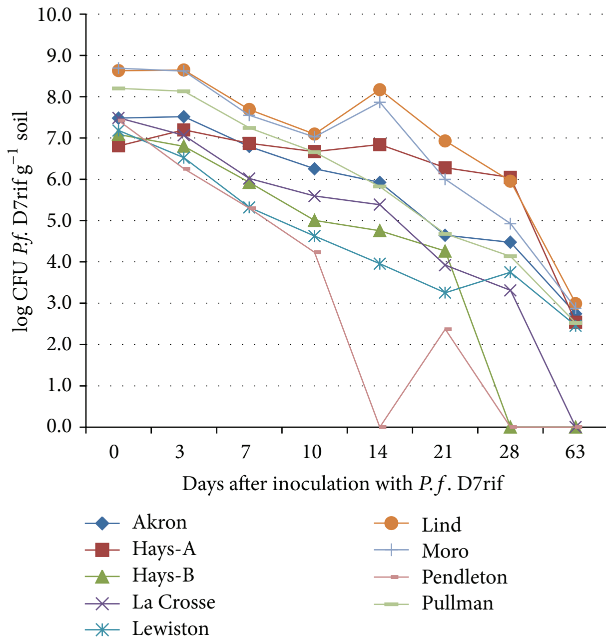

Figure 1: Recovery of *P.f.* D7rif in log CFU g^{-1} from nine different soils over a 63-day incubation at 20°C. A difference greater than log 0.5 indicates significant difference at $P \leq 0.05$.

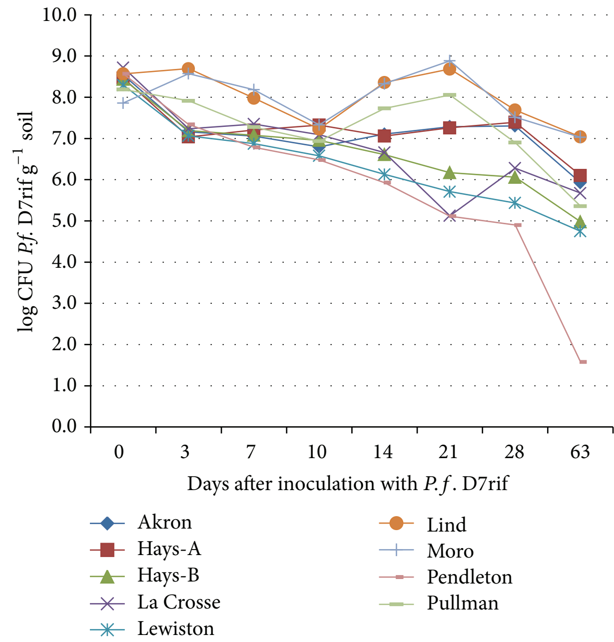

Figure 2: Recovery of *P.f.* D7rif in log CFU g^{-1} from nine different soils over a 63-day incubation at 5°C. A difference greater than log 1.0 indicates significant difference at $P \leq 0.05$.

by soil type and incubation temperature at each sampling time ($P < 0.05$). Soil moisture content only affected *P.f.* D7rif recovery at Day 7 sampling. Interactions between soil type and incubation temperature could only be detected at Day 3 sampling time. There were no interactions between soil type and soil moisture content in this study.

When averaged over moisture treatments, *P.f.* D7rif survival dropped off dramatically for all soil types at the 20°C incubation temperature (Figure 1). Counts of *P.f.* D7rif were higher than log 8.5 CFU g^{-1} soil for Lind, WA, and Moro, OR, at the beginning of the study and declined to just under log 3.0 CFU g^{-1} soil by Day 63. In all soils there was a decline in *P.f.* D7rif from Day 3 to Day 10, but the Lind, Moro, and Hays, KS-"A" soils showed an increase at Day 14 before resuming a decline in populations to Day 63. By Day 28, no *P.f.* D7rif was recovered from Pendleton, OR, or Hays, KS-"B" soils, and by Day 63 there was no recovery from the La Crosse, WA, soil. At 5°C *P.f.* D7rif recovery from five of the nine test soils was greater than log 5.5 colony forming units (CFU) g^{-1} soil after 63 days (Figure 2), a level which is adequate for suppression of downy brome under field or range conditions (Kennedy, unpublished data). Recovery of *P.f.* D7rif from the 10°C incubation temperature was slightly lower than at 5°C; however, relationships among soils were similar with the greatest recovery from the Lind and Moro soils and the least recovery from the Pendleton soil (data not shown).

At each incubation temperature, *P.f.* D7rif recovery was highest from Lind and Moro soils (Table 4), and both of those soils also had the smallest slope values at the 5°C and 10°C temperatures. *P.f.* D7rif recovery was lowest from

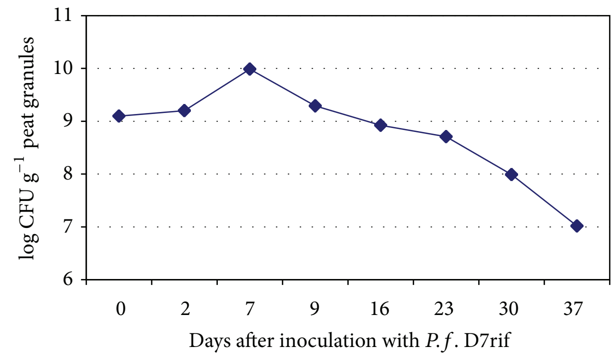

Figure 3: Recovery of *P. fluorescens* D7rif from peat granules after 37-day incubation.

the Pendleton soil at the 5°C and 10°C temperatures. The steepest slope values over the 63-day incubation were also found for the Pendleton soil at those temperatures. Three soils, Hays-"B", La Crosse, and Pendleton, had no *P.f.* D7rif recovery after 63 days. The steepest slope for 20°C was from the Hays-"B" soil. Regression analysis indicated that over the 63-day incubation period, there were no correlations between *P.f.* D7rif survival and any of the soil textural or chemical properties (R^2 all <0.09; data not shown).

3.2. Survival of P.f. D7rif in Peat Granules. The initial level of *P.f.* D7rif in peat granules was log 9.1 CFU g^{-1} peat granules (Figure 3). *P.f.* D7rif was recovered at a peak level of log 10 CFU g^{-1} peat granules on Day 7 before declining to

Table 4: *Pseudomonas fluorescens* D7rif survival and slope of trendline after 63-day incubation at 5, 10, and 20°C, averaged across moisture contents.

Soil	5°C		10°C		20°C	
	log CFU g^{-1}	Slope	log CFU g^{-1}	Slope	log CFU g^{-1}	Slope
Akron, CO	5.93	−0.0257	5.74	−0.0297	2.75	−0.0781
Hays, KS-"A"	6.11	−0.0236	5.05	−0.0498	2.54	−0.0713
Hays, KS-"B"	4.98	−0.045	5.04	−0.0419	0	−0.1212
La Crosse, WA	5.68	−0.038	4.55	−0.0481	0	−0.1172
Lewiston, ID	4.75	−0.047	4.86	−0.0408	2.46	−0.0659
Lind, WA	7.04	−0.0208	6.49	−0.0292	2.99	−0.0895
Moro, OR	7.03	−0.0173	6.45	−0.0293	2.88	−0.0947
Pendleton, OR	1.58	−0.1008	3.16	−0.074	0	−0.1106
Pullman, WA	5.36	−0.0391	4.60	−0.0544	2.52	−0.0932

a level of log 7.0 CFU g^{-1} peat granules on Day 37, the last sampling time of the study.

4. Discussion

Rifampicin-resistant strains of beneficial bacteria have been utilized by other researchers to monitor survival of the introduced organism in soil or on plant tissue [12, 13] and a number of years after application in the field [14]. The ultimate goal of our research was to show that the promising biological control organism *Pseudomonas fluorescens* strain D7 can survive long enough in soil to colonize downy brome seed and roots and inhibit growth of the weed until beneficial crop and rangeland plants can gain a competitive advantage. We set out to demonstrate this using *P.f.* D7rif, a rifampicin-resistant strain of the organism.

Nine soils from different locations were chosen for the incubation study to determine whether soil texture or some other soil property influences survival of *P.f.* D7rif and if that might impose limitations on the range of locations where *P.f.* D7 could be used as a biological control agent against downy brome. Silt loam, silty clay loam, and loam soils were represented in this study (Table 2). In their study of microbial communities across land-use types, Lauber et al. [15] found that soil pH and texture significantly affected the composition of the bacterial community, in correlation with the percent silt and clay. Soils with higher clay content are considered to be more protective of the soil microbial biomass due to their ability to protect microbes from drying and maintain soil pH and because clays might adsorb compounds unfavorable for microbial growth [16]. In this study, the Hays, KS-"B" soil had the highest clay content; however, this did not translate into greater bacterial survival (Figures 1 and 2). In agricultural soils from The Netherlands, Garbeva et al. [17] found that plant species and soil type were important factors influencing the rhizosphere bacterial community, including total *Pseudomonas*. They showed that total numbers of bacteria, including *Pseudomonas,* were higher when grown in the presence of plants. *Pseudomonas fluorescens* D7 was originally isolated from wheat roots [5] and survived in high enough numbers to inhibit weed growth by colonizing the rhizosphere. Survival of *P.f.* D7rif in our study may have increased in the presence of plant roots.

One of the greatest obstacles to success of a biological control agent, and its subsequent commercialization, is the ability to survive in soil. Survival of rhizobacteria as biological control agents is also impacted by adequate colonization of weed seeds and roots and microbial ecology in the rhizosphere of weeds and other host plants [18]. Survival of *P.f.* D7rif over the 63-day incubation study dramatically decreased as incubation temperature increased from 5°C to 20°C. Other scientists have seen decreased survival of *Pseudomonas* spp. at increased soil temperatures of 25 to 35°C [18] and 35°C [19]. *Pseudomonas fluorescens* survival has also been shown to be sensitive to changes in soil water potential when comparing saturated and unsaturated (−0.06 MPa) soil conditions [20]; however, we did not see any significant differences in *P.f.* D7rif survival between the two soil water potentials tested in this study. It is possible that the two water potentials tested here (0.03 and 0.10 MPa) were not distinct enough to lead to significant differences in bacterial survival and recovery.

Quality of soil organic matter and crop residue affect soil microbial community functions such as enzyme activity, respiration, and N mineralization [21]. In general, higher microbial populations are found in soils with more crop residue [22], where soils have been amended with organic or inorganic fertilizers [23] and soil organic C is higher [24]. There was no correlation between high organic matter and *P.f.* D7rif recovery in the incubation study. Soil organic matter in these soils ranged from just over 1% for the Lind soil to 5% for the Lewiston soil; however, the Lind soil showed the highest survival rate, while the soil with the highest organic matter, Lewiston, was among the soils with the lowest survival rate.

Soil from Pendleton had the lowest pH of any of the test soils (5.2), and survival of *P.f.* D7rif was lowest at Pendleton for each of the incubation temperatures (Table 4). He et al. [25] found the lowest microbial taxonomy and metabolic diversity in forest soils with the lowest pH. Hartel et al. [19], in a similar incubation study comparing survival of a wild type *Pseudomonas putida* with its genetically altered mutant, found that the soil environment affected survival of the two organisms differently. They showed reduced survival

of both wild type and mutant isolates at higher temperatures, although the wild type was better able to withstand the extremes of high temperature or low soil moisture content. The more acidic soils were among the lowest for bacterial survival in that study [19].

Soil nutrient content is one of the factors contributing to survival of bacterial biological control agents in soil. Krumins et al. [26] found that the bacterial community in sandy, forest soils changed in response to nitrogen added as NH_4NO_3, and they concluded that in sandy soils the bacterial community was more sensitive to N additions than the fungal community. In our incubation study, there was no correlation between overall *P.f.* D7rif recovery and either high or low soil NH_4-N or NO_3-N. When NH_4-N and NO_3-N values were combined for each soil (data not shown), two of the soils with the lowest total N (Lind, Moro) had the highest *P.f.* D7rif overall survival. In their study of P and K deficient red soils (Ultisols and Oxisols) in China, He et al. [25] showed that pH and soil organic carbon were the most significant soil characteristics affecting soil microbial communities, but other factors such as nitrogen, cation exchange capacity, soil moisture, and compaction affected soil microbial communities. Vasquez et al. [27] found that greater soil N led to greater competitiveness of cheatgrass in range settings, and so the nutrient status of soil may affect the ability of desired plant communities to compete and reduce cheatgrass. We did not find correlations between soil nutrients or chemical properties and *P.f.* D7rif survival in this study.

Gu and Mazzola [28] found that production environment may influence the ability of bacterial biological control agents to survive and function. Limited carbon environments may lead to improved survival of organisms when exposed to other stresses, including extreme temperature [29]. Varying growth conditions of the *P.f.* D7rif inoculum was not part of this experiment and *P.f.* D7rif cultures were grown using standard methods [5, 30]; however, limiting C and exposing isolates to greater stress may be a way to improve survival under field conditions.

In the peat granule study, the initial concentration of *P.f.* D7rif was comparable to that of the beginning *P.f.* D7rif population recovered from the Lind soil, which had the highest survival in the incubation study. After 28 days of incubation, recovery of *P.f.* D7rif from the Lind soil was about log 6.0 CFU g^{-1} soil, while recovery from the peat granules was 1.0 log higher at log 7.0 CFU g^{-1} peat after 37 days. Elzein et al. [31] found that the mycoherbicide *Fusarium oxysporum* “Foxy 2” could be encapsulated in a pesta formulation to improve shelf life, and Kohlschmid et al. [32] found that a combination of alginate pellets and pesta granules formulated with the *Fusarium oxysporum* isolate FOG was more efficacious and reliable in controlling the parasitic weed branched broomrape (*Phelipanche ramosa* (L.) Pomel) than the control under field conditions. Peat granules are used for commercial inoculants for legumes, and encapsulation in peat granules or some other material to protect bacterial biological control agents seems promising to allow for the greatest survival, efficacy and success in biological weed control.

5. Conclusions

P.f. D7rif recovery from soil following the 63-day incubation was highest at the lowest incubation temperature (5°C), and in soils from Lind, Washington, and Moro, Oregon. The ability of *P.f.* D7 to survive in soil and colonize plant roots and residue at the cooler temperatures experienced in the Western United States during the fall corresponds favorably to the most optimal temperature for application of the bacterial biological control agent to control downy brome in winter wheat. Survival of *P.f.* D7rif varied among the nine soils, but we found no clear relationships between soil textural and chemical characteristics and *P.f.* D7rif survival. The application of *P.f.* D7rif encapsulated in peat granules appears promising for protecting the bacteria from harsh environmental conditions, allowing for the greatest survival and biological weed control.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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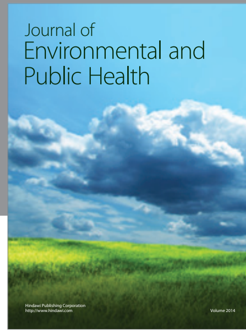
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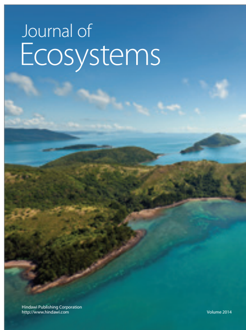
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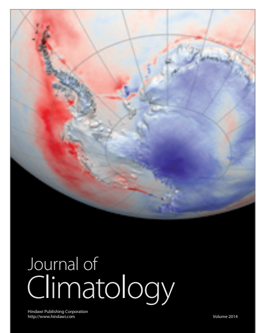
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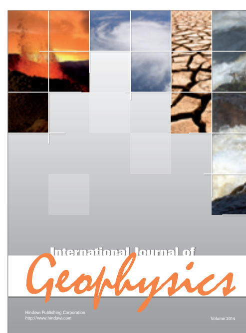
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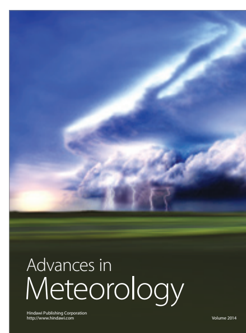
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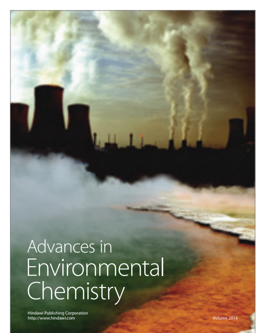
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