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Weed-Suppressive Bacteria Fail to Control *Bromus tectorum* Under Field Conditions☆

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ABSTRACT

The exotic winter annual grass *Bromus tectorum* L. (downy brome or cheatgrass) infests millions of hectares of western rangelands. Weed-suppressive bacteria (ACK55 and D7 strains of *Pseudomonas fluorescens* Migula 1895) have been shown to reduce *B. tectorum* populations in eastern Washington. Unfortunately, outside of Washington, little is known about the efficacy of these or other weed-suppressive bacteria. We used Petri-plate and plant-soil bioassays to test effects of ACK55 and D7 on *B. tectorum* from Montana and Wyoming. We also tested effects of ACK55 on *B. tectorum* at six field sites in Montana and one in Wyoming. *P. fluorescens* reduced *B. tectorum* germination and root and shoot lengths in Petri-plates but had no effect on plants during growth chamber plant-soil bioassays or field experiments. *Bromus arvensis* L. (field brome or Japanese brome), a species similar to *B. tectorum,* was prevalent at two of our sites, and ACK55 was ineffective against *B. arvensis* as well. Our findings contribute to a growing body of evidence that the ACK55 and D7 strains of *P. fluorescens* are not reliable tools for controlling *B. tectorum* in the Northern Great Plains, Central Rocky Mountains, and elsewhere.

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Introduction

The exotic winter annual grass *Bromus tectorum* dominates millions of hectares in the western United States (Duncan et al., 2004). Herbicides are commonly used management tools that can drastically reduce *B. tectorum* populations, but populations often rebound quickly (e.g., Morris et al., 2009; Owen et al., 2017), though other studies have reported more lasting effects (Sebastian et al., 2017). Various microbes (bacteria, fungi, viruses) are also under investigation as potential tools for combatting *B. tectorum* invasions, and some have been reported to suppress winter annual grasses (e.g., Mazzola et al., 1995; Kennedy et al., 2001; Meyer et al., 2001; Beckstead et al., 2014; Harding and Raizada, 2015; Kennedy, 2018). In contrast with herbicides, these microbes have generally not moved from the research and development phase to widespread use and have had limited market availability.

The soil-borne bacteria *Pseudomonas fluorescens* proliferates in fall and winter (Ibekwe et al., 2010), and some strains negatively affect the biomass, fecundity, and cover of *B. tectorum* (Kennedy et al., 1991; Kennedy, 2018). In related research, *P. fluorescens* suppressed *B. tectorum* root growth with minimal direct effects on other plant species (Kennedy et al., 2001; Kennedy, 2018). *P. fluorescens* survived best in soil (Mollisols) from cropland in Washington and Oregon but survived well in other Mollisols from seven other cropland sites (Stubbs et al., 2014). Unfortunately, Stubbs et al. (2014) did not sample soils from rangeland, especially rangeland with *B. tectorum* infestations.

To date, most *P. fluorescens* research has occurred in Petri-plate and growth chamber environments (e.g., Johnson et al., 1993;

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Ibekwe et al., 2010). However, Kennedy et al. (1991) reported the D7 strain of *P. fluorescens* controlled *B. tectorum* in two of three wheat fields in eastern Washington. Conversely, Reynecke (2012) reported no effect of D7 on *B. tectorum* at a rangeland site in eastern Washington. A patent by Kennedy (2017) reported field trial results for the ACK55 strain of *P. fluorescens* at six sites (four in Washington, one in Idaho, and one in Oregon) and peak control of *B. tectorum* cover occurred 4 yr post application. Similarly, Kennedy (2018) reported ACK55 suppressed *B. tectorum* at five rangeland sites in Washington. Despite the reported successes with D7 and ACK55, papers also stated, "… attempts to duplicate these [D7, Kennedy et al., 1991] results in subsequent field studies have had limited success …" (Tranel et al., 1993) and "Not shown here are data from countless [ACK55] field studies that showed no weed inhibition …" (Kennedy, 2018). Also, there is mounting evidence that the efficacy of *P. fluorescens* heavily depends on soil moisture and temperature at the time of application (Kennedy, 2018) and also on *B. tectorum* genetics Kennedy et al., 2001.

Though the results of field trials have been mixed, popular press articles (Boxall, 2013; Campbell, 2015; Solomon, 2015) have implied that the putative weed-suppressive bacteria hold promise for *B. tectorum* control. In response, land managers have purchased and applied *P. fluorescens* to > 10 000 ha (Campbell, 2015). This has prompted warnings about the lack of peer-reviewed research in Montana, Wyoming, and elsewhere (Kosto, 2018).

To address these concerns, we tested the effects of *P. fluorescens* in a series of experiments. Petri-plate experiments tested the effects of *P. fluorescens* (D7 and ACK55) on germination and root and shoot lengths of *B. tectorum* germinants from Montana and Wyoming. Similarly, we used a plant-soil bioassay conducted in a growth chamber and tested effects of *P. fluorescens* (D7 and ACK55) on root and shoot biomass of *B. tectorum* seedlings from Montana and Wyoming. We predicted that *P. fluorescens* would primarily reduce root length and root biomass (e.g., Johnson et al., 1993; Ibekwe et al., 2010). We also used a coordinated distributed field experiment to test the effect of ACK55 applications (2014) over 4 yr (2015 – 2018) on percent cover of *B. tectorum* in six sites in Montana and one in Wyoming. The field experiment also tested effects of ACK55 on *B. arvensis* cover at two sites in Montana. We predicted peak weed-suppression 4 yr post application (Kennedy, 2018, fig. 4).

Methods

Petri-Plate Bioassay

The Agricultural Research Service Culture Collection provided *P. fluorescens* strains D7 (NRRL #B-18293) and ACK55 (NRRL #B-50848). After working with several sources of *P. fluorescens* strain ACK55, we found most produced two distinct bacterial colony types (Fig. S1; available online at https://doi.org/10.1016/j.rama.2019.07.006). We refer to the two colonies as "ACK55-large" and "ACK55-small." The Petri-plate and plant-soil bioassays tested the effects of D7, ACK55-large, and ACK55-small.

We used a 4 × 2 factorial experiment with 3 replications for each treatment combination (i.e., 24 total plates). The treatments were bacteria (control, D7, ACK55-large, ACK55-small) and seed source (Miles City, Montana and Bill, Wyoming).

D7, ACK55-large, and ACK55-small colonies were transferred via sterile loop to 5 mL of Sands and Rovira broth (Sands and Rovira, 1970) and shaken at 22°C for 48 h, allowing them to reach stationary phase. For use as a Petri-plate inoculant, each strain's stationary phase culture was then grown to midlog phase. To attain midlog phase, King's B broth (King et al., 1954) was inoculated and the mixture was shaken at room temperature for 31 h. To determine colony forming units (CFU) per mL at the time *B. tectorum* seeds were exposed to the strains, we serially diluted the midlog phase cultures and counted bacteria colonies on Petri-plates. CFU per mL were 4.37×10^8 (D7), 4.10×10^8 (ACK55-large), and 1.70×10^7 (ACK55-small).

Methods for testing effects of the strains follow those of Kennedy (2016). We pipetted 1 mL of each midlog phase culture onto 100 × 15 mm Petri-plates covered with solidified sterile water agar (0.9%). Control plates received 1 mL of sterile King's B broth. After allowing liquid to soak into agar for 3 – 4 h, we placed 10 *B. tectorum* seeds onto the agar's surface of each plate. Seeds were collected in 2018 from rangeland in Miles City, Montana and Bill, Wyoming, and were surface-sterilized to remove bacteria and fungi (Appendix S1; available online at https://doi.org/10.1016/j.rama.2019.07.006). We repeated the bioassay without sterilizing the seeds and achieved similar results (*results not shown*). Plates were incubated for 7 d at 15°C. We recorded germination and measured root and shoot length of all germinants.

Plant-Soil Bioassays

For each *P. fluorescens* strain/type (i.e., D7, ACK55-large, and ACK55-small), we conducted a separate 2 × 2 factorial experiment with five replications. The treatments were bacteria (control and bacteria) and seed source (Montana and Wyoming) (2 [bacteria treatments] × 2 [seed sources] × 5 = 20 pots per strain/type). The methods resembled similar studies (Kennedy et al., 1991; Johnson et al., 1993). Another pot experiment tested the effects of two soil types (loam, sand) and three soil inoculant treatments (soil filtrate [control], filtrate and ACK55, and ACK55) on biomass of *B. tectorum* from Montana (supplemental appendix contains the methods and results (see Appendix S1).

Liquid bacteria cultures were created and used in inoculating pots. For the D7 strain, three flasks containing 150 mL of *Pseudomonas* minimal salts (PMS) broth (Bolton et al., 1989) were inoculated with two D7 colonies per flask (24-h-old colonies of medium B NPC agar (Sands and Rovira, 1970) incubated in the dark at 23°C) and then flasks were incubated on a bench top shaker (24 h, 22°C, 180 rpm). ACK55 grew slower than D7 and required different inoculation steps and longer incubations. For ACK55-large, three flasks of PMS broth (110 mL per flask) were inoculated (48-h-old colonies) and incubated for 48 h. For ACK55-small, three flasks of PMS broth (100 mL per flask) were inoculated with 10 ACK55-small colonies per flask (72-h-old colonies) and incubated for 72 h. To determine CFU per mL at the time *B. tectorum* seedlings were exposed to the strains, we serially diluted the liquid cultures and counted bacteria colonies on Petri-plates. CFU per mL were 1.39×10^8 (D7), 2.40×10^8 (ACK55-large), and 2.00×10^7 (ACK55-small). Rates approximated those of prior bioassays (Kennedy et al., 1991; Kennedy, 2018).

Table 1
Analysis of variance for a Petri-plate bioassay testing effects of four bacterial treatments (control, D7, ACK55-large, and ACK55-small) and two seed sources (Montana and Wyoming) on germination rate and on shoot and root length of *Bromus tectorum* seedlings.

Variable	Model effects	F	df	*P*
Germination rate	Bacteria	60.3232	3	< 0.01
	Seed source	0.1212	1	0.73
	Bacteria × seed	5.0505	3	0.01
Shoot length	Bacteria	40.6606	3	< 0.01
	Seed source	7.3877	1	0.02
	Bacteria × seed	1.2431	3	0.33
Root length	Bacteria	42.6071	3	< 0.01
	Seed source	2.9131	1	0.11
	Bacteria × seed	3.4167	3	0.05

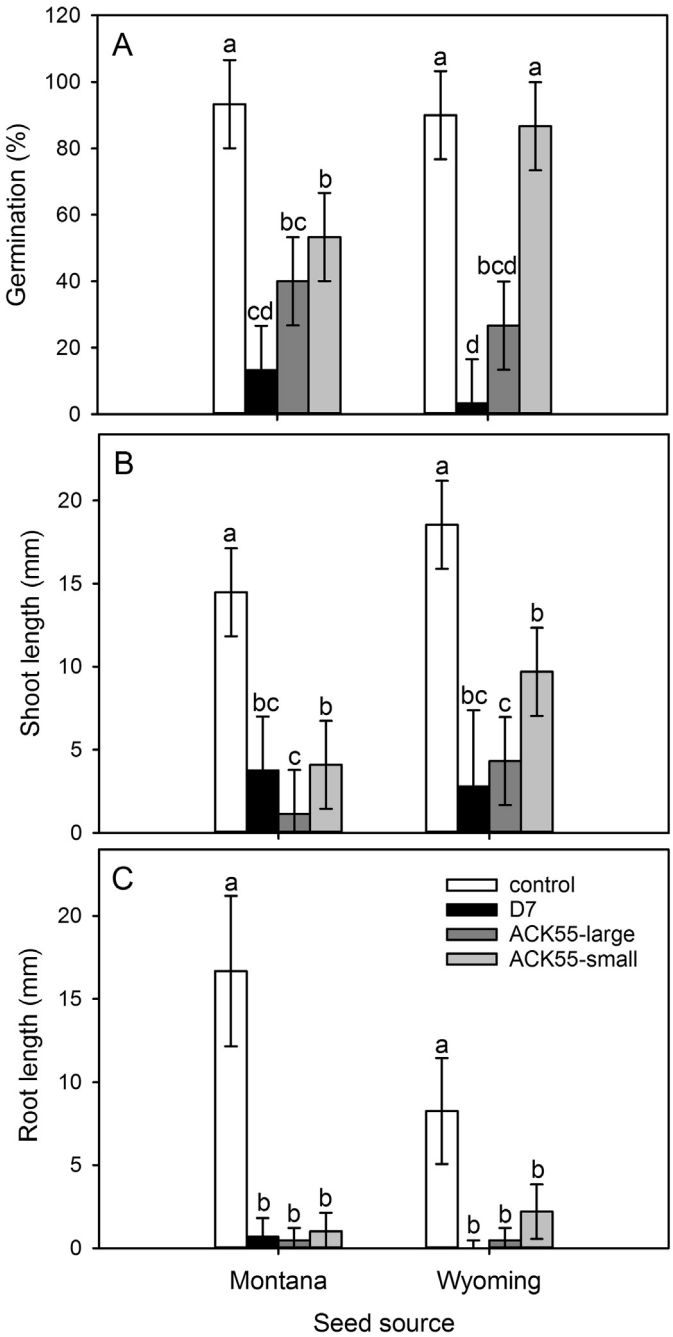

Figure 1. Means and 95% confidence intervals quantifying effects of three strains of *Pseudomonas fluorescens* on *Bromus tectorum* in a Petri-plate bioassay. Tukey-Kramer differences for **A**, bacteria and seed source treatments and **B** and **C,** bacteria shown by lowercase letters.

Seed material was the same as that used in the *Petri-plate bioassay*. To ensure adequate seed germination, seed was germinated in the Fort Keogh Livestock and Range Research Laboratory's greenhouse, Miles City, Montana. Seed was sown onto surface-sterilized trays containing autoclaved silica sand. Trays were placed on a bench with a misting system regulated by an Electronic Leaf (Phytotronics, Inc., Earth City, MO) and supplied with water purified using reverse osmosis (RO).

Pots (surface area 100 cm^2, 750 mL) were filled with sieved (6.5 mm), autoclaved field soil (Eapa fine loam, frigid Aridic Argiustolls); watered until saturated with RO water; allowed to drain for 16 h in a growth chamber (21°C, dark conditions); and weighed to determine pot weight at field capacity. Pots were assigned to bacterial treatments and planted with three *B. tectorum* seedlings. Before planting, seedling roots were dipped into several mL of a bacterial or control (i.e., PMS broth without bacteria) solution. After transplanting, we pipetted 3 mL per pot of bacterial or control solution onto the soil surrounding seedlings. Pots were then placed into a growth chamber with a 10-h light period at 10°C. Three times a week, RO water was added to replace evaporation losses, and pots were rerandomized. After 30 d in the growth chamber, seedlings were harvested and roots were separated from the soil by hand washing. Shoot and root samples were dried (60°C) to constant weight and weighed.

Coordinated Distributed Field Experiment

In 2014, ACK55 and control treatments were applied at eight sites (seven in Montana and one in Wyoming) to 5 × 5 m plots arranged in a randomized complete block design with eight replications per site, except for two western Montana sites with four replications. The primary aim was to test the effect of ACK55 on percent cover of *B. tectorum*. A secondary aim was to test effects of ACK55 on a similar exotic invasive species, *Bromus arvensis. B. arvensis* was present at six of eight total sites, but only two sites in eastern Montana had *B. arvensis* in all plots. (Note: one of eight sites contained *B. arvensis* but not *B. tectorum*). Overall, four sites were in the Central Rocky Mountains (western Montana) and four in the Northern Great Plains (eastern Montana and Wyoming, Fig. S2; available online at https://doi.org/10.1016/j.rama.2019.07.006).

Dr. Ann C. Kennedy at the US Department of Agriculture–Agricultural Research Service's (USDA-ARS) Northwest Sustainable Agroecosystems Research Lab in Pullman, Washington had Environmental Protection Agency approval to use ACK55 in field experiments and to treat ≤5 acres per yr. In 2014, 10 g (50 × 10^9 cells per g) of freeze-dried ACK55 (NRRL B-50848) were transferred to K.O.R. at USDA-ARS's Fort Keogh Livestock and Range Research Laboratory.

To carry out a distributed experiment, cooperators were given instructions on site selection; replications (4 or 8); plot size and space between plots (≥ 1 m); sprayer calibration; mixing and applying ACK55; and monitoring. In brief, each cooperator was given 0.1125 g of ACK55, enough to treat nine plots. Each aliquot was mixed with 6.3 L of nonchlorinated (or degassed) water (8.93 × 10^5 cells per mL). Each plot received 0.7 L of treatment solution

Table 2
Analysis of variance for a plant-soil bioassay testing effects of two bacterial treatments (control, *Pseudomonas fluorescens*) and two seed sources (Montana and Wyoming) on biomass of *Bromus tectorum* seedlings. A separate bioassay was conducted for each *P. fluorescens* strain/type (D7, ACK55-large, ACK55-small).

Bacteria strains	Variable	Treatments	F	df	*P*
D7	Shoot	Bacteria	2.04	1	0.17
		Seed source	9.88	1	< 0.01
		Bacteria × seed	1.06	1	0.32
	Root	Bacteria	2.27	1	0.15
		Seed source	0.02	1	0.88
		Bacteria × seed	2.55	1	0.13
ACK55-large	Shoot	Bacteria	1.28	1	0.27
		Seed source	16.17	1	< 0.01
		Bacteria × seed	0.00	1	0.97
	Root	Bacteria	2.52	1	0.13
		Seed source	3.34	1	0.09
		Bacteria × seed	1.25	1	0.28
ACK55-small	Shoot	Bacteria	0.02	1	0.90
		Seed source	1.73	1	0.21
		Bacteria × seed	1.33	1	0.27
	Root	Bacteria	0.08	1	0.78
		Seed source	0.23	1	0.64
		Bacteria × seed	1.00	1	0.33

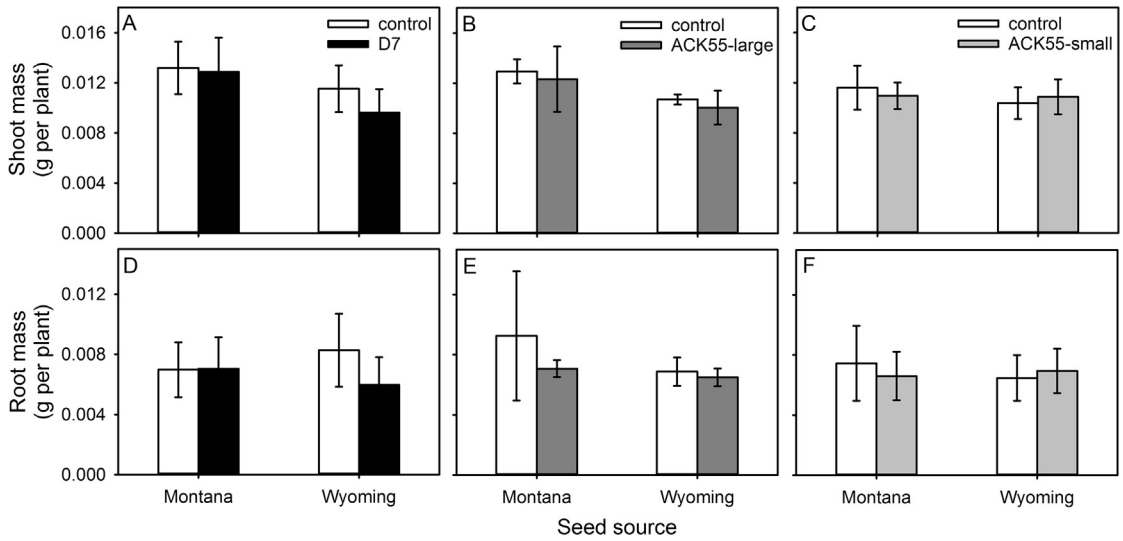

Figure 2. A–F, Means and 95% confidence intervals quantifying effects of three strains of *Pseudomonas fluorescens* on *Bromus tectorum* in plant-soil bioassays. No treated values differ from control values at the 5% level of confidence.

(2.5×10^7 cells per m^2, rate recommendation from Kennedy, A.C., *personal communication* [Oct. 23, 2014]) or a similar amount of water from a separate sprayer (e.g., 7.6 L Home and Garden Sprayer, ACE Hardware, Oak Brook, IL). Preferred conditions for treating plots were cold temperatures (< 16°C), imminent precipitation, and minimal wind to prevent drift (Kennedy, 2017). Sites were treated from November 26 through December 26, 2014. Temperatures at the time of application across sites ranged from -3.9 to 18.3°C. Because of logistical constraints, one site (Wyoming) was treated when ambient temperatures exceeded recommended levels. Because precipitation was not expected at this site, plots were watered with an equivalent of 5 mm of rainfall. Livestock were not permitted to graze the areas for ≥ 1 yr.

Annually from 2015 to 2018, percent cover of *B. tectorum* and *B. arvensis* was estimated separately by species at seed head emergence in quadrats, except one site in western Montana, which estimated cover from 2014 (pretreatment) to 2018 and two other sites in western Montana that combined cover of both species.

Data Analysis

For the Petri-plate bioassay, we tested the effects of bacteria treatment and seed source on germination rate and mean root and shoot length per plate with 2-way analysis of variance (ANOVA, JMP 12.1.0, SAS Institute, Cary, NC). Root length was square root–transformed to homogenize variance. We used Tukey-Kramer analyses to investigate differences among treatments. Where we detected treatment × seed source interactions, we used linear contrasts to compare differences between seed sources within treatments.

For the plant-soil bioassay, data of individual seedlings per pot were aggregated (i.e., averaged) before analysis. Two-way ANOVAs

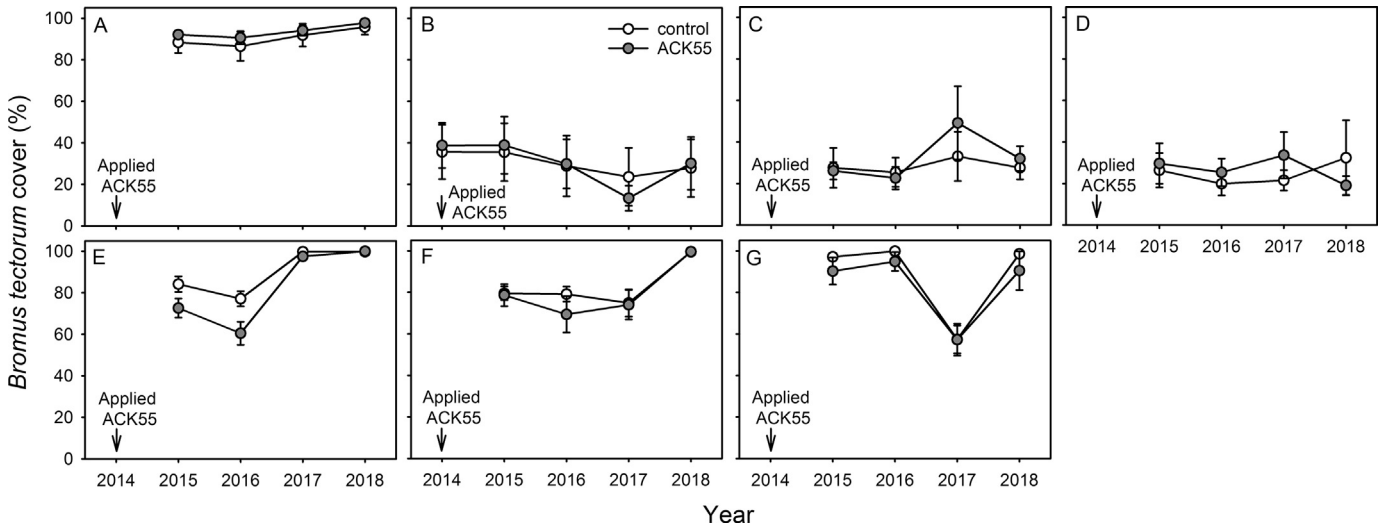

Figure 3. Means and 95% confidence intervals quantifying effects of the ACK55 strain of *Pseudomonas fluorescens* on *Bromus tectorum* cover at seven sites. Panels represent sites from **A–D,** Central Rocky Mountains and **E–G,** Northern Great Plains. **Panels A–G** correspond with sites 1 through 7, respectively, in Fig. S2; available online at https://doi.org/10.1016/j.rama.2019.07.006. Few treated values differ from control values at the 5% level of confidence, except the 2015 and 2016 data in **Panel E.**

were used to test the effects of bacteria and seed source on root and shoot biomass. A separate ANOVA was performed for each bacteria strain/type and response variable with R (R Development Core Team, 2011).

For the field experiment, conclusions about *B. tectorum* were the same whether or not we included two sites in western Montana that combined cover of *B. tectorum* and *B. arvensis*. Therefore, we present our combined analysis of all seven sites with *B. tectorum*. Cover data were logit-transformed and analyzed with a linear mixed model having fixed effects for site, year and treatment and random effects for site by year, site by treatment, year by treatment, and site by year by treatment. Significance of the treatment main effect was evaluated from the corresponding confidence interval, and significance of interactions involving treatment was evaluated using posterior predictive *P* values (Gelman et al., 2014). The model was fit with a program written in Fortran (Intel Corporation, 2013).

Results

Petri-Plate Bioassay

All three bacterial strains reduced germination for both seed sources except for ACK55-small, which did not significantly reduce germination of the Wyoming seed source (Table 1, Fig. 1A). Across seed sources, D7 reduced germination by 91% and ACK55-large by 64%. The ACK55-small reduced Montana seed germination by 43% but did not appreciably reduce Wyoming seed germination.

Across bacteria treatments, shoot lengths were greater for the Wyoming than Montana seed source (see Table 1, Fig. 1B). D7, ACK55-large, and ACK55-small reduced shoot length by 79%, 83%, and 58%, respectively (see Fig. 1B). D7, ACK55-large, and ACK55-small reduced root length by 73–100% (see Fig. 1C).

Plant-Soil Bioassay

Opposite to the *Petri-plate bioassay*, *B. tectorum* from Montana tended to have greater shoot biomass than *B. tectorum* from Wyoming (Table 2, Fig. 2A and 2B). None of the bacteria strains reduced root ($P \geq 0.13$) or shoot ($P \geq 0.17$) biomass (see Table 2, Fig. 2).

Coordinated Distributed Field Experiment

Except for one site, we found no evidence that ACK55 reduced *B. tectorum* ($P \geq 0.24$, Fig. 3). For a site in eastern Montana (see Fig. 3E), ACK55-treated plots had less *B. tectorum* cover than controls in 2015 and 2016. However, no differences were detected in 2017 and 2018, thereby suggesting these initial differences were either ephemeral effects (Kennedy, 2018) or reflected pretreatment differences. In addition, we found no evidence ACK55 affected *B. arvensis* cover ($P \geq 0.50$, Fig. S3; available online at https://doi.org/10.1016/j.rama.2019.07.006). A concern was that ACK55 might colonize control plots over the study period. We found no evidence that *Bromus* spp. cover declined through time in the control plots, indicating ACK55 did not affect *Bromus* spp. in control plots.

Discussion

In Petri-plate experiments, the ACK55 and D7 strains of *P. fluorescens* inhibited *B. tectorum* from Montana and Wyoming, but under field conditions, ACK55 provided no appreciable control of *B. tectorum* and *B. arvensis* at eight sites. Therefore, while ACK55 is capable of suppressing *B. tectorum* from Montana and Wyoming under highly controlled conditions, ACK55 as we applied it did not reliably control *B. tectorum* in Montana and Wyoming rangelands.

Several studies found that *P. fluorescens* D7 (e.g., Johnson et al., 1993; Kennedy et al., 2001) and ACK55 (Kennedy, 2017, 2018) negatively impacted *B. tectorum* growing in pots with field soil in the growth chamber; our experiments are the first to report no effect of *P. fluorescens* D7 and ACK55 on potted *B. tectorum* in growth chamber experiments. This was surprising because *P. fluorescens* reportedly impacted *B. tectorum* over a range of temperatures and soil moisture levels (Johnson et al., 1993), and growth chamber conditions did not vary dramatically between our study and previous studies. Moreover, ACK55 proved incapable of suppressing *B. tectorum* under multiple soil types (loam, sand) and inoculant treatments (soil filtrate [control], filtrate and ACK55, and ACK55) (see Appendix S1; Fig. S4; available online at https://doi.org/10.1016/j.rama.2019.07.006). It is unclear what factors prevented *P. fluorescens* from impacting *B. tectorum* in the growth chamber. Possible explanations involve soil structural/chemical properties and competing microbes introduced to pots from the seeds or from water or air.

While *P. fluorescens* reduced *B. tectorum* in certain experiments in three studies with sites in Idaho, Oregon, and Washington (Tranel et al., 1993; Kennedy, 2017, 2018), journal articles describing two of the studies allude to experiments that were excluded from the articles due to a lack of effect. Specifically, Tranel et al. (1993) indicate, "… attempts to duplicate these [D7, Kennedy et al., 1991] results in subsequent field studies have had limited success …" and Kennedy (2018) writes, "Not shown here are data from countless [ACK55] field studies that showed no weed inhibition …" Moreover, Reynecke (2012) found no effect on *B. tectorum* 1 yr after treatment at a rangeland site in Washington—no effect was also detected 3 and 4 yr after treatment (Brown, R., *personal communication* [Sept. 22, 2016]). Research in Idaho also failed to detect effects of *P. fluorescens* strains three years after treatment (Lazarus and Germino et al., 2019)

Implications for Range Management

Popular press articles have suggested that particular bacteria are a useful tool for controlling *B. tectorum* (e.g., Campbell, 2015), and land managers across the western United States have begun applying the bacteria to large *B. tectorum* – infested areas. However, it is becoming increasingly clear that the bacteria often fail to control *B. tectorum*. Our Montana and Wyoming experiments contribute to a growing body of research indicating currently available strains of *P. fluorescens* are at present not a reliable tool for controlling *B. tectorum*. Given the expense of acquiring and applying bacterial formations, we recommend against using currently available bacterial strains for controlling *B. tectorum* without prior controlled and replicated studies that include monitoring to ensure effectiveness at a given site.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rama.2019.07.006.

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