AGRICULTURAL RESEARCH FOUNDATION
FINAL REPORT
FUNDING CYCLE 2014 – 2016

TITLE: Effects of *Pseudomonas fluorescens* WH6 on annual bluegrass emergence in established perennial ryegrass.

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SUMMARY:

Pseudomonas fluorescens WH6, a rhizosphere bacterium isolated from Willamette Valley soils, produces a naturally-occurring herbicide. This herbicide, a Germination Arrest Factor (GAF) selectively and irreversibly arrests the germination of a weedy grass species (including annual bluegrass) without significantly affecting established grass seedlings or mature plants. However, no field experiments have been initiated that evaluate the ability of the bacterium to control weeds in situ. The primary objective of this research is to assess the pre-emergence effects of *P. fluorescens* WH6 on annual bluegrass germination in established turfgrass stands. Preliminary findings from the research initiated in the fall of 2013 determined that a single application of *P. fluorescens* just prior to seeding provided no effect on annual bluegrass populations. Therefore, in 2014 a new research project was initiated to determine if weekly applications of Pseudomonas fluorescens WH6 effectively controlled annual bluegrass.

OVERALL OBJECTIVE:

- Evaluate the pre-emergence effects of *Pseudomonas fluorescens* WH6 on weed germination in established perennial ryegrass.

EXPERIMENT 1: FALL 2013 TO FALL 2014

Field research was initiated at the Lewis-Brown Horticulture Farm, Corvallis, OR on March 7, 2013.

Objectives:

1. Evaluate the pre-emergence effects of *Pseudomonas fluorescens* WH6 applied at varying rates on annual bluegrass germination in an established perennial ryegrass stand.

2. Evaluate the pre-emergence effects of *Pseudomonas fluorescens* WH6 applied at varying rates on white clover germination in an established perennial ryegrass stand.

3. Evaluate the effects of *P. fluorescens* WH6 on established perennial ryegrass health and vigor

Procedure:

The experimental area was a 60 ft. x 35 ft. stand of well-established perennial ryegrass, with individual 5 ft. x 5 ft plots. Treatments included applications of *P. fluorescens* WH6 at 192, 384, 576 ml of solution per 25 ft^2, as well as a control treatment. Each treatment was arranged in a randomized complete block design with six replications. *Pseudomonas fluorescens* WH6 was grown in sterile culture at the USDA Forage Seed and Cereal Research Laboratory in Corvallis, OR, and then applied at a concentration of 6.3 x 10^{11} colony forming units (c.f.u.) per ml of potassium substituted (PMS) solution. The control treatments received applications of the PMS solution without *P. fluorescens* WH6.

Applications at rates ranging from 0 to 576 ml of *P. fluorescens* WH6 solution per 25 ft^2 were made twice annually on October 8, 2013 and March 11, 2014. On October 8, 2013, just prior to the application of the *P. fluorescens* WH6 solution all plots were seeded with annual bluegrass at a rate of 1 lb. per 1,000 ft^2 (germination rate >96%). The perennial ryegrass was prepped before seeding by reducing the mowing height from 2" to 5/8". The perennial ryegrass and seed were raked with a plastic leaf rake immediately after seeding to move the seed off the leaf blades and onto the soil. *Pseudomonas fluorescens* WH6 applications were repeated on a per plot basis using the above rates in October 2013.

The perennial ryegrass stand was maintained at a 2.0 inch mowing height, with clippings returned. Regular applications of a complete fertilizer were applied at a rate of 3 lbs. N per 1,000 ft^2 annually. Irrigation was adjusted according to the environmental conditions, with 0.15 inches applied daily during the peak of the summer, June, July and August.

Response Variables:

A 0.3 m^2 grid with intersects on a 2.5 cm spacing was used to quantify the percent annual bluegrass cover (0-100%). The number of intersects containing annual bluegrass (a) was divided

by the total number of intersects (b=25) to determine the percent of annual bluegrass cover (a/b*100 = % annual bluegrass). The same methods were also used to quantity the percent of white clover.

Significant Accomplishments: Experiment 1

Findings from the research initiated in the fall of 2013 determined that *P. fluorescens*, when applied in a single application just prior to seeding, had no deleterious effects on annual bluegrass populations, observed in the following spring and fall (Table 1). This preliminary study determined that the annual bluegrass seed applied on Oct 8, 2013 did not begin to germinate until 10 days after seeding, and that the germination of the seed was indeterminate, meaning that seeds germinated at different times over a 2 month period from October to December. Given this, it is not surprising that a single application of *P. fluorescens* WH6 was not able to control annual bluegrass germination. In order to be an effective biocontrol agent, *P. fluorescens* WH6 must be in contact with the seed during the germination process. Therefore, in this scenario, it is unlikely that the bacterium was present over the two-month germination process, and that any treatment effects were therefore minimized.

As suspected, prior to the initiation of this trial, no effects on established perennial ryegrass or white clover populations were observed (Table 1; white clover results only).

Table 1: Effects of *Pseudomonas fluorescens* rate on percent annual bluegrass cover (0-100%) within a well-established stand of perennial ryegrass at Lewis-Brown Farm in Corvallis, OR, assessed in April and Oct of 2014.

			Percent annual		Percent	
			Apr-14	Oct-14	Apr-14	Oct-14
Source of variation	**Num DF**	**Den DF**	***Pr* > F**			
Pseudomonas fluorescens rate	5	25	NS	NS	NS	NS

Solution* rate (ml/25 ft^2) + *Pseudomonas fluorescens* concentration	Percent annual bluegrass (0-100%)				Percent white clover (0-100%)			
	Apr-14		Oct-14		Apr-14		Oct-14	
192 ml + 6.3 x 10^{11} c.f.u.** per ml	22.7	a	24.5	a	13.9	a	11.1	a
384 ml + 6.3 x 10^{11} c.f.u. per ml	21.3	a	23.2	a	22.2	a	6.5	a
576 ml + 6.3 x 10^{11} c.f.u. per ml	15.3	a	23.6	a	23.2	a	11.6	a
192 ml without *P. fluorescens*	13.4	a	30.1	a	21.3	a	7.0	a
384 ml without *P. fluorescens*	23.6	a	22.2	a	19.9	a	10.7	a
576 ml without *P. fluorescens*	16.2	a	29.6	a	14.4	a	4.2	a

*****Potassium substituted (PMS) solution was applied March 11, 2013 and October 8, 2013.
******Colony forming units (c.f.u.) per ml of PMS solution

EXPERIMENT 2: FALL 2014 TO FALL 2015

In response to the observations made during the initial trial, a new research project was initiated at the Lewis-Brown Horticulture Farm, Corvallis, OR on Dec 5, 2014.

Objective:

Evaluate the pre-emergence effects of *Pseudomonas fluorescens* WH6 on annual bluegrass germination in an established perennial ryegrass stand when delivered using repeated applications over 1 ½ month period.

Procedure:

The experimental area is a 300 ft^2 stand of perennial ryegrass established in the spring of 2014. Individual plots are 5 ft. x 5 ft., and the experimental design is a randomized complete block design with six replications. Treatments include sequential application of *P. fluorescens* WH6 in comparison to a control treatment.

On Dec 12, 2014 all plots were seeded with annual bluegrass at a rate of 1 lb. per 1,000 ft^2 (germination rate >96%). On the day of seeding, the first of many *P. fluorescens* WH6 applications was made. The *P. fluorescens* WH6 bacterial solutions were applied at a rate of 384 ml of solution per 25 ft^2 (a concentration of 7.5 x 10^{11} colony forming units per ml of 0.4x potassium substituted PMS amended with 10 mM arginine), a untreated control was also applied. On Dec 19, 2014, and Jan 1, 2015 the second and third subsequent treatment were made to the same plots (Table 2). The same application rate of *P. fluorescens* WH6 was maintained through the duration of the study.

The perennial ryegrass stand used for this project is maintained at a 2.0 inch mowing height, with clippings returned. Regular applications of a complete fertilizer were applied at a rate of 3 lbs. N per 1,000 ft^2 annually. Irrigation was adjusted according to the environmental conditions, with 0.15 inches applied daily during the peak of the summer, June, July and August.

Response Variables:

A 0.3 m^2 grid with intersects on a 2.5 cm spacing was used to quantify the percent annual bluegrass cover (0-100%). The number of intersects containing annual bluegrass (a) was divided by the total number of intersects (b=25) to determine the percent of annual bluegrass cover (a/b*100 = % annual bluegrass).

Significant Accomplishments: Experiment 2

Twenty eight days after the third *P. fluorescens* WH6 application, plots treated with bacterial solution had an average annual bluegrass population of 8.8% while the control treatments had 10.6%, a reduction of 1.8% provided by the *P. fluorescens* WH6 (Table 2). In the fall of 2015, plots treated with the bacterial solution had an average annual bluegrass

population of 33.4%, while the control treatments had 39.0%, a reduction of 5.6% provided by the *P. fluorescens* WH6. While these findings document slight difference the results were not significantly different at a 0.05 level of probability. Therefore, further investigation into the optimum application method (i.e. solution rate, colony forming unit rate, solution amendments) is required before adequate control of annual bluegrass is realized.

Benefits & Impacts:

Results suggested that *P. fluorescens* WH6 in the application methods utilized in Experiment 2 provide a slight reduction in annual bluegrass germination. However, in efforts to maximum the effects and successful application of this alternative weed control method a series of new objectives are currently being explored (Experiment 3, see below).

Table 2: Effects of repeated *Pseudomonas fluorescens* applications on percent annual bluegrass cover (0-100%) within a well-established stand of perennial ryegrass at Lewis-Brown Farm in Corvallis, OR, assessed January 29, 2015 and Oct 28, 2015.

Source of variation	**DF Num**	**DF Den**	**...Pr > F...**
Pseudomonas fluorescens	1	2	NS

	percent annual bluegrass (0-100%)	
Treatment	29-Jan-15	28-Oct-15
*Pseudomonas fluorescens**	8.8 a	33.4 a
Control	10.6 a	39.0 a

*****Three subsequent applications of *Pseudomonas fluorescens* [384 ml of solution per 25 ft^2 with a concentration of 7.5 x 1011 colony forming units (c.f.u.) per ml of 0.4x potassium substituted solution amended with 10 Mm Arginine medium (final pH=6.8)] were applied on Dec 12, Dec 19, 2014, and Jan 1, 2015.

**Means within columns followed by the same letter are not significantly different according to least significant difference at the 0.05 probability level.

EXPERIMENT 3: DECEMEBER 2014 TO PRESENT

To determine the minimum number of *P. fluorescens* WH6 applications for optimum control an additional study was initiated on Dec 3, 2014. The purpose of the trial was to determine if multiple applications of actively growing WH6 controlled the germination of annual bluegrass seed sown onto the surface of soil flat, which had been placed into the field. In this experiment, we sprayed the soil surface containing the weed seed four times with WH6-inoculum (or with a sterile culture medium as a control) on two- week intervals over a two-month period, in parallel with the field experiment described in Experiment 2. Once annual bluegrass germination occurred in the flats that were sprayed with the sterile media control solution, the WH6 sprayed and control flats were transferred to the greenhouse for observation. After the seedlings matured, the total number of seedlings were counted and the data analyzed. In the flats treated with WH6, the germination of ABG was significantly ($p<0.005$) reduced (Fig.1). Molecular assays that were designed to specifically detect WH6 indicated that WH6 bacteria were present in the inoculated flats, but not in the control (Fig. 2). The nearly 50% reduction in germination of ABG in the inoculated flats is the first indication that we will be able to control ABG in soils with live cultures of WH6 in a field setting. Although these preliminary results are promising, repeated biological sprays and a 50% reduction in weed germination are not practical solutions for PNW grass seed growers.

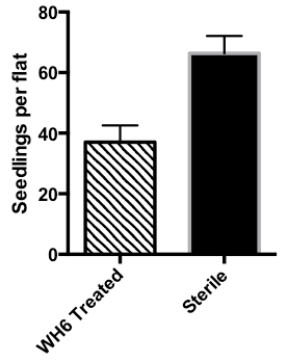

Fig. 1. Seedlings of Poa annua in flats treated with WH6 inoculated or with sterile media.

We believe that there are several ways to improve WH6-based biocontrol of ABG. Therefore, the objective of this proposal is to increase the ability of WH6 to control ABG germination by optimizing culture conditions and by adding adjuvants to our inoculating sprays. Based on the results presented in Figure 3 and Figure 4, in 2016 the Oregon Seed Council agreed to fund a follow up study with the following objectives.

Aim 1) Determine the minimum number of applications that are required to control ABG germination.

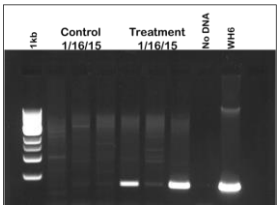

Fig. 2. The presence of *P. fluorescens* WH6 in control flats or those treated with WH6-inoculated sprays post

Aim 2) Determine if the addition of adjuvant increases the survival of WH6 in soils.

Aim 3) Determine if the addition of growth substrates increases the survival of WH6 in soils.

Aim 4) Determine the short-term durability of WH6-based biocontrol activity in soils.

Experiments that support these aims are underway.

Anticipated Benefits & Impacts:

FVG and its analogs represent a new class of potentially effective herbicides to control grassy weeds in perennial crops. However, chemical synthesis of FVG has been proven difficult. Likewise, the structure of the molecule renders fermentation-based production strategies too costly. Biocontrol therefore represents one option for utilizing this novel herbicide, and for transferring the knowledge that we have gained to PNW grass seed growers. We expect that the initial optimization of biocontrol studies will inform subsequent field studies which may lead into a commercial application of an FVG-producing bacterium for the biological control of grassy weeds in the Willamette Valley.

Current Funding:

This research is funded by the Oregon Seed Council ($5,000) and the research is currently ongoing.