
An assessment of environmental conditions for control of downy brome by *Pseudomonas fluorescens* D7

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Abstract: *Purpose:* We evaluated the conditions that favoured *Pseudomonas fluorescens* strain D7 (*P.f.* D7) growth and inhibition of downy brome.

Design/methodology/approach: Tn5 mutagenesis and a competitive assay were used to isolate mutants of *P.f.* D7. Isolates were screened for polysaccharide production and toxin response. Seven mutants were tested under varying pH, temperature and water potential and characterised using Random Amplified Polymorphic DNA analysis.

Findings: Temperature, pH, and water potential did not affect weed suppression in bioassays, except at 37°C and with NaCl.

Originality and/value: Understanding the genetics of *P.f.* D7 will help in the development of successful weed biocontrol systems.

Keywords: biological control; *Pseudomonas fluorescens* strain D7; Tn5 mutagenesis; downy brome.

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1 Introduction

Downy brome (*Bromus tectorum* L.) is a winter annual grass weed in eastern Washington State, USA, that grows vigorously during early spring before winter wheat (*Triticum aestivum* L.) resumes growth, giving the weed a competitive advantage for limited resources during the growing season. *Pseudomonas fluorescens* strain D7 (*P.f.* D7; NRRL B-18293) is a naturally occurring soil bacterium that is being explored as a potential biological control agent against downy brome. *Pseudomonas fluorescens* strain D7 is a non-pathogenic, rod-shaped, Gram-negative soil bacterium. *P.f.* D7 selectively inhibits downy brome and some *Bromus* spp. and does not harm non-target plants (Kennedy et al., 1991). In agriculture, *P.f.* D7 may play a beneficial role as a biological control agent with benign interaction with wheat roots and inhibitory effects on downy brome roots (Kennedy and Kremer, 1996).

In field experiments, Kennedy et al. (1991) obtained reductions in downy brome biomass of 18–54% when *P.f.* D7 was applied to the soil surface. This in turn reduced downy brome competition and enhanced wheat yield above a level, which would have been seen in non-weedy check plots. In similar studies, when wheat was interplanted with downy brome, increases in wheat biomass were obtained with different bacterial treatments relative to untreated controls, suggesting that the different rhizobacteria strains diminished the competitive abilities of downy brome (Mazzola et al., 1995). Therefore, the use of this biocontrol agent may be very effective in selectively controlling downy brome in winter wheat systems. Biocontrol options will give greater flexibility

in winter wheat production where selective chemical herbicides for grass weeds are limited or when available are expensive and residual, which limits the ability to rotate crops.

Current control methods for downy brome include herbicides, tillage and crop rotation (Ball et al., 2004). A large proportion of downy brome seed germinates in the autumn and develops root mass from November to March (Thill et al., 1984), coinciding with establishment of winter wheat. However, downy brome can resume root growth before winter wheat in the early spring, giving it a competitive advantage for limited soil moisture (Thill et al., 1984). It is at this period that *P.f.* D7 may colonise downy brome roots and produce root-suppressive compounds that may limit the competitive abilities of downy brome.

In many biocontrol systems, the effectiveness of the agent can be limited by many factors such as loss of ecological competence, inadequate production of active compound, and differences in root colonisation by the bacterial strains (Weller, 1988; Weller and Thomashow, 1993; de Luna et al., 2005). Root colonisation by rhizobacteria may also be limited by different soil factors, such as soil water content and temperature (Loper et al., 1985; Schippers et al., 1987; Johnson et al., 1993). Phytotoxicity of microorganisms is plant-specific and cultivar specific (Schippers et al., 1987). The ability of *P.f.* D7 to suppress the growth of downy brome is due to the production of a phytotoxin (Tranel et al., 1993). In the rhizosphere, this bacterium has shown the ability to adapt to extreme soil environmental conditions and still suppress downy brome (Johnson et al., 1993).

The plant-suppressive compound from this bacterium has been isolated and partially purified (Gurusiddaiah et al., 1994), and is known to be a complex of chromopeptides and peptides, fatty acid esters and a lipopolysaccharide matrix; however, little is known about the genetic basis of phytotoxin production in *P.f.* D7. Early work (Xu and Gross, 1988; Kennedy et al., 1992) on related strains used mutation to disrupt the genes responsible for the expression of toxin in root inhibition, root colonisation, and rhizosphere competitiveness. These studies suggested that there are several genes involved in phytotoxin production, and these genes are located at various loci on the chromosome. They concluded that the toxin might be a secondary metabolite whose synthesis requires a number of enzymes and may also depend on a number of regulatory genes.

This study looks at the genetic analysis of chromosomal determinants of toxin production in *P.f.* D7 relative to their response to different environmental factors. Previous studies had indicated that toxins produced by certain strains of *Pseudomonas fluorescens* can inhibit the growth of some strains of *E. coli* (Bolton et al., 1989; Fredrickson and Elliott, 1985). A toxin-resistant *E. coli* donor strain (WA803) was used to investigate the relationship between toxin production and competitiveness. A suicide plasmid vector pGS9 (Selvaraj and Iyer, 1983) was used to generate random Tn5 insertion mutants of strain WA803, which were used to deliver the transposon. The objectives of these studies were to compare the competitive abilities of the toxin-negative (Tox^-) strains and the wild type *P.f.* D7 under different environmental conditions, and to compare diversity and genetics of wild type and mutant strains.

2 Materials and methods

2.1 Strains, plasmids and isolation of mutants

Pseudomonas fluorescens strain D7 was obtained from the rhizoplane of winter wheat and downy brome in southeastern Washington State (Kennedy et al., 1991). *Pseudomonas fluorescens* D7rif (*P.f.* D7rif) is a spontaneous, rifampicin-resistant mutant of strain *P.f.* D7 that was selected for use in determining survival of the biocontrol agent in field and laboratory studies. The Tox^- mutants were obtained in the following manner. The recipient, *P.f.* D7, was grown at 25°C to mid-log phase in King's Medium B broth (KB; King et al., 1954). The donor, *E. coli* strain WA803, was grown at 37°C to mid-log phase in Luria and Bertani (LB) broth (Maniatis et al., 1982) supplemented with kanamycin (Km) at 25 mg L⁻¹ to maintain Tn5. Transposon Tn5 was delivered by the suicide plasmid, pGS9 (Selvaraj and Iyer, 1983), contained in WA803. This plasmid, pGS9, is composed of a 15A replicon and the N conjugative system. Cells were centrifuged at 12,000X g for 10 min and washed twice with LB broth. This process removes excess polysaccharides from recipient cells and exposes sex pili. Sterile saline (NaCl, 0.08%) was used for the final wash of donor cells.

The donor, *E. coli* strain WA803, was chosen because of its resistance to the toxin produced by *P.f.* D7. For mating, equal volumes (0.1 mL each) of the donor and recipient cells were spotted onto nitrocellulose membranes placed on LB agar and incubated for 26 h at 28°C. Mutants were isolated by aseptically placing membranes into centrifuge tubes containing LB broth. After incubation, cells were washed off membranes with *Pseudomonas* Minimal Salts (PMS) broth (Bolton et al., 1989), serially diluted, spread onto PMS agar plates supplemented with vitamin-free casamino acids (200 mg L⁻¹) and Km (100 mg L⁻¹) and incubated at 25°C for three days for the selection of transconjugants. Controls for the donor or the recipient cells were treated similar to the mixed cell suspension. Transconjugants were checked for streptomycin resistance (Sm^r) and chloramphenicol sensitivity (Cm^s). In addition, Kanamycin-resistant (Km^r) transconjugants were stabbed into PMS agar containing Km (100 mg L⁻¹) either with or without vitamin-free Casamino Acids to screen for amino acid auxotrophs. Tox^- activities were tested by growing mutants in PMS broth and performing a bioassay with downy brome seed on agar medium (Kennedy et al., 1991). Reversion frequencies of Tn5 insertions were determined for auxotrophic Tn5 mutants by growing single colonies in 25 mL of nutrient broth-yeast extract medium (NBY, Vidaver and Davis, 1988) at 25°C. Frequencies of reversion to prototrophy were determined by differences in viable cell counts. Growth characteristics were determined by culturing *P.f.* D7 on Sands and Rovira agar medium (Sands and Rovira, 1970) and in *Pseudomonas* minimal salts medium (Bolton et al., 1989). Colony morphology of the mutants was similar to that of the parental strains, except in the cases of the Pol^- mutants.

Pseudomonas fluorescens strain D7-wild type (Recipient), D7-rifampicin resistant (*P.f.* D7rif), strains E669 (Tox^+), 5C57 (Pol^+), 5E59 (Tox^-), 6E103 (Tox^-), E6-46 (Tox^-), 5C12 (Pol^-) and 5C4 (Pol^-) were tested in culture condition studies and in DNA studies (Table 1). Strains 5E59, 6E103 and E6-46 were selected from among 1911 Tn5 mutants based on their Tox^- activity on the weed downy brome and their similarity in growth habit to *P.f.* D7. Strains 5C12 and 5C4 were selected among the Tn5 mutants based on their lack of polysaccharide production (Pol^-). Strain E669 was selected from among the

Tn5 mutants; however, it maintained the downy brome inhibitory effect and strain 5C57 was a Tn5 mutant similar to the wild type in polysaccharide production.

Table 1 Characteristics of *Pseudomonas fluorescens* strain D7, mutants and other bacterial strains used in this study

Strain	Characteristics	Plant effect	Reference
<i>Pseudomonas fluorescens</i> strain D7	Wild type	Downy brome inhibitory	Kennedy et al. (1991)
<i>Pseudomonas fluorescens</i> strain D7rif	Spontaneous, rifampicin resistant	Downy brome inhibitory	
<i>Escherichia coli</i> WA803	Met ⁻ , Thi ⁻ Donor		Wood (1966)
Plasmid pGS9	P15A replicon, Cm ⁻ , Km ⁻ (Tn5)		Selvaraj and Iyer (1983)
<i>Pseudomonas fluorescens</i> strain E669	Tn5 mutant of <i>P.f.</i> D7	Downy brome inhibitory; Tox ⁺	This manuscript
<i>Pseudomonas fluorescens</i> strain 5C57	Tn5 mutant of <i>P.f.</i> D7, polysaccharide produced; Pol ⁺	Downy brome inhibitory; Tox ⁺	This manuscript
<i>Pseudomonas fluorescens</i> strain 5E59	Tn5 mutant of <i>P.f.</i> D7	Non-inhibitory to downy brome; Tox ⁻	This manuscript
<i>Pseudomonas fluorescens</i> strain 6E103	Tn5 mutant of <i>P.f.</i> D7	Non-inhibitory to downy brome; Tox ⁻	This manuscript
<i>Pseudomonas fluorescens</i> strain E6-46	Tn5 mutant of <i>P.f.</i> D7	Non-inhibitory to downy brome; Tox ⁻	This manuscript
<i>Pseudomonas fluorescens</i> strain 5C12	Tn5 mutant of <i>P.f.</i> D7, reduced polysaccharide; Pol ⁻	Downy brome inhibitory; Tox ⁺	This manuscript
<i>Pseudomonas fluorescens</i> strain 5C4	Tn5 mutant of <i>P.f.</i> D7, reduced polysaccharide; Pol ⁻	Downy brome inhibitory; Tox ⁺	This manuscript

2.2 Polysaccharide production

The electron microscope was used to observe polysaccharide envelope of mutants and the wild type that were cultured in KB medium for 48–72 h. Cells were harvested and centrifuged at 8000 g for 30 min and stained as described by Gurusiddaiah et al. (1994). Each isolate was rated on its polysaccharide production compared with the wild type. Single colonies of the mutants were tested in King's B medium for polysaccharide production, inhibition of bacterial growth and colour differences compared with the wild type.

2.3 Environmental analyses

Isolates of the following stock cultures were used for the experiment: *P.f.* D7, *P.f.* D7rif, E669 Tox⁺, 5C57 Pol⁺, 5E59 Tox⁻, 6E103 Tox⁻, E6-46 Tox⁻, 5C12 Pol⁻ Tox⁺ and 5C4 Pol⁻. Each of the Pol isolates exhibited Tox⁺ behaviour. *P.f.* D7, D7rif, and mutants were grown in PMS broth for up to 72 h at various pH, temperature and water potential levels. Cultures were initially grown in PMS broth for 24 h, centrifuged, washed and resuspended in PMS broth to a density of 10⁵ Colony Forming Units (CFU) mL⁻¹. Tubes (12 × 100 mm) containing PMS broth were inoculated with the 24 h washed cultures. Cells were grown at 22°C for up to 72 h in PMS broth on a rotary shaker at 250 rpm. At 0, 4, 8, 24, 28, 32, 48, and 72 h, growth was assayed spectrophotometrically (600 nm). Every 24 h, culture samples were withdrawn for serial dilution and cell enumeration on KB agar. Generation times were calculated as the population doubling time in mid-log phase.

For pH studies, broth was buffered after autoclaving to pH 5, 6 or 7 using 0.1 M citric acid and 0.2 M Na₂HPO₄ (Costilow, 1981) and monitored throughout the study to maintain the pH to close to the initial pH value. *P.f.* D7, *P.f.* D7rif and mutants were grown at pH 5, 6, 7 for up to 72 h at 22°C. To test temperature response, cultures were incubated at 4, 15, 24, and 37°C in PMS broth at pH 7.0. To study response to osmotic potential, PMS broth was amended with 29.2 g NaCl or 200 g polyethylene glycol L⁻¹ (PEG; 3350 mol. wt.; Sigma Chemical Co., St. Louis, MO) to change osmotic potential to approximately -2.4 MPa (Harris, 1981). Unless otherwise stated, the media was pH 7 and the bacteria were grown at 22°C for each of the above experiments. Each of the experiments described earlier was conducted twice, with three replications of each isolate-medium treatment. Data from one of the two experiments is presented.

2.4 DNA isolation and Southern blot analysis

Chromosomal and plasmid DNA were isolated from mutants and the wild type by using the Qiagen DNA and plasmid preparation kits (Qiagen, USA). DNA was restricted with EcoRI at 37°C for 3 h. The restricted DNA was separated through a 0.8% (w/v) agarose gel and blotted onto Hybond-N+ nylon membrane (Amersham Int., Little Chalfont, Bucks, UK). The blot was prehybridised and hybridised at 65°C in 7% (w/v) Sodium Dodecyl Sulphate (SDS), 1% (w/v) Bovine Serum Albumin (BSA) and 0.5 M phosphate buffer. Purified plasmid (pGS9) was labelled with [³²P] using the random hexamer labelling procedure (Feinberg and Vogelstein, 1983; Storlie et al., 1998). Blots were prehybridised for 3–5 h before the probe was added and then hybridised for 12–18 h. After hybridisation the blot was washed in 0.1% SDS at 65°C. With 500–1000 counts per minute of radiation, the blot was placed on autoradiography film for 2–5 days.

2.5 Strain differentiation

Chromosomal DNA was isolated as described earlier from strains that were Tox⁺ and Tox⁻, as well as from strains that were positive and negative for polysaccharide production (Pol⁺, Pol⁻). Polymerase chain reaction was done by using Ready-To-Go-Random Amplified Polymorphic DNA (Ready-To-Go-RAPD) analysis according to the manufacturer's instruction (Amersham Pharmacia Biotech, Piscataway, NJ) with the

RAPD primer 6-(5'-d[CCCGTCAGCA]-3'), after testing six other primers for strain differentiation. The PCR products were separated on a 2% agarose gel in a TBE buffer. Ethidium bromide stained gels were photographed using Polaroid film (Waltham, MA).

2.6 Root suppression as a function of culture condition

The root-suppressive activity of culture fluids or supernatants at 24, 48 or 72 h was determined using the bioassay for downy brome-suppressive compounds (Kennedy et al., 1991). Cultures from isolates *P.f.* D7, *P.f.* D7rif, 5E59 Tox⁻ and 6E103 Tox⁻ at each pH, temperature and water potential treatment were centrifuged at 8,800X g for 15 min. The supernatant (1 mL), which contained less than 10⁶ cells mL⁻¹ was dispensed onto 18 mL of cooled 1.0% water agar and allowed to soak into the agar. Sterile PMS broth was added to control plates. The supernatant alone was used in the agar plate bioassay because we were interested in the activity of the secondary metabolites produced by *P.f.* D7 in inhibiting seed germination and root growth, not in causing infection of the seeds or roots (Fredrickson and Elliott, 1985). Ten downy brome seeds were planted on the agar surface and incubated in continuous darkness at 15°C. Root lengths were measured after five days. Each of the bioassay experiments was conducted twice, with three replications of each isolate-medium treatment. Root length of treated and control plants were measured after five days. The degree of downy brome root inhibition was expressed as a percent of the control.

2.7 Data analysis

Data were analysed with SAS (SAS/STAT User's Guide, 1999) using the general linear models procedure, including ANOVA and the Tukey's test ($P \leq 0.05$) (Steel et al., 1997). Log transformation of bacterial population data was performed prior to analysis. DNA fingerprints obtained from the RAPD banding patterns on 2% agarose gels were photographed and digitised using ImageMaster Labscan (Amersham-Pharmacia Biotech, Uppsala, Sweden). The lanes were normalised to contain the same amount of total signal after background subtraction. The gel image was straightened and aligned using ImageMaster 1D Elite 3.01 (Amersham-Pharmacia Biotech, Uppsala, Sweden) and analysed to give a densitometric curve for each gel. Band positions were converted to R_f values between 0 and 1 and profile similarity was calculated for the total number of lane patterns (Ibekwe et al., 2001; Ibekwe and Grieve, 2004). A dendrogram was constructed by using the Unweighted Pair Group method with Mathematical Averages (UPGMA) to determine lane similarities.

3 Results and discussions

3.1 Environmental factors affecting the survival and growth of *Pseudomonas fluorescens* strain D7

When grown at pH 5, 6 or 7, generation times for the *P.f.* D7-wild type ranged from 1.5 h to 1.6 h (Table 2). After 48 h at pH 5, 6, or 7, *P.f.* D7 increased to populations of 8.9, 7.8 and 8.3 log₁₀ CFU mL⁻¹, respectively (Table 2). Generation times for the three Tox⁻ mutants ranged from 1.7 h to 3.8 h and these mutants only attained populations of

6.0–7.8 log₁₀ CFU mL⁻¹ after 48 h. *P.f.* D7 had a generation time of 1.5 h at pH 5.0, and the Tox⁻ mutants had generation times of 3.6 h or greater. The growth of the Pol⁻ or Pol⁺ variants was similar to the wild type except for a longer generation time (2.2 h) at pH 7. Tox⁻ mutants grew at a faster rate at pH 6 than at pH 5 and 7. At pH 6, there were no significant differences in the generation time among the wild type and the mutants; however, the population numbers at 48 h were significantly lower for two of the Tox⁻ mutants (Table 2). Two Tox⁻ mutants (6E103 and 5E59) only reached a maximum growth of 6.8–7.0 log₁₀ CFU mL⁻¹ for the three pH levels studied (Table 2). *P.f.* D7rif growth was similar to the *P.f.* D7 for all but the generation time at pH 5. The Pol⁻ mutants were similar to the wild type at pH 5 and 6.

Table 2 Generation time and cell numbers of batch-cultured *Pseudomonas fluorescens* D7, *P.f.* D7rif, and strains E669 (Tox⁺), 5C57 (Pol⁺), 5E59 (Tox⁻), 6E103 (Tox⁻), E6-46 (Tox⁻), 5C12 (Pol⁻), and 5C4 (Pol⁻) at 22°C in PMS broth buffered to pH 5, 6 and 7

<i>D7 variant</i>	<i>Generation time (h)</i>			<i>Log₁₀ CFU^a mL⁻¹ at 48 h</i>		
	<i>pH 5</i>	<i>pH 6</i>	<i>pH 7</i>	<i>PH 5</i>	<i>pH 6</i>	<i>pH 7</i>
<i>P.f.</i> D7	1.5a ^b	1.6a	1.5a	8.9a	7.8a	8.3a
<i>P.f.</i> D7rif	2.1b	1.7a	1.4a	8.4a	8.2a	8.7a
<i>P.f.</i> E669	1.5a	1.6a	1.5a	8.9a	8.0a	8.3a
<i>P.f.</i> 5C57	1.2a	1.5a	1.5a	8.2a	8.0a	8.3a
<i>P.f.</i> 5E59	3.6c	1.7a	2.2b	6.7b	7.0b	6.0b
<i>P.f.</i> 6E103	3.8c	1.8a	1.9ab	6.3b	6.8b	6.5b
<i>P.f.</i> E6-46	3.6c	1.6a	1.9ab	6.7b	7.8a	7.0ab
<i>P.f.</i> 5C12	1.5a	1.8a	2.2b	8.7a	8.0a	7.7a
<i>P.f.</i> 5C4	1.3a	1.6a	2.2b	8.6a	8.5a	8.0a

^aCFU: Colony Forming Units.

^bWithin each column, means followed by different letters are significantly different at $P \leq 0.05$.

The Tox⁻ mutants derived from this study were less tolerant to changes in pH and were more restricted in their distribution over a wide range of pH. The rate of growth for the wild type and mutants at different pH ranges suggests that the *P.f.* D7 can grow in a relatively wide environmental pH range. Our data suggest that the wild type is highly plastic and is capable of tolerating a wide range of pH values. The results of this study are advantageous because for adequate weed control, a high cell density of active biocontrol agent is required to survive under the varying soil pH levels that may be found in wheat producing regions.

The wild type and mutants varied in their response to temperature (Table 3). At 4°C, there was an increase in the populations of the *P.f.* D7, E669, and 5C57 to 7.0 log₁₀ CFU mL⁻¹ at 48 h. The Tox⁻ mutants 5E59, 6E103 Tox⁻, 6E103 and E6-46 were undetectable at 48 h at 4°C and did not recover for the test period at that low temperature. The Tox⁻ strain 6E103 had a long generation time at 4°C and 37°C. The Pol⁻ mutants had lower populations of 5.1–6.0 CFU mL⁻¹ at 4°C after 48 h. Growth rates of the wild type and D7rif were similar at 15, 24 and 37°C; however, the growth rates of the Tox⁻ mutants and one Pol⁻ variant were significantly higher at 24°C than at 4°C. When the temperature

was increased to 37°C, both wild type and mutant inoculant survived less than 48 h. This supports the findings of Johnson et al. (1993) that *P.f.* D7 growth is inhibited by high temperatures.

Table 3 Generation time and cell numbers of batch-cultured *Pseudomonas fluorescens* D7, *P.f.* D7rif, and strains E669 (Tox⁺), 5C57 (Pol⁺), 5E59 (Tox⁻), 6E103 (Tox⁻), E6-46 (Tox⁻), 5C12 (Pol⁻), and 5C4 (Pol⁻) and incubated in PMS broth at 4, 15, 24 and 37°C

D7 variant	Incubation temperature (°C)							
	Generation time (h)				Log ₁₀ CFU ^a mL ⁻¹ at 48 h			
	4	15	24	37	4	15	24	37
<i>P.f.</i> D7	1.8a ^b	1.7a	1.7a	2.4a	7.0a	6.8a	7.3a	<1.0a
<i>P.f.</i> D7rif	2.1b	1.9a	1.8a	2.4a	<1.0b	5.3b	7.7a	<1.0a
<i>P.f.</i> E669	1.6a	1.6a	1.7a	2.4a	7.0a	6.9a	7.0a	<1.0a
<i>P.f.</i> 5C57	1.6a	1.6a	1.6b	2.1a	7.2a	7.8a	8.1a	<1.0a
<i>P.f.</i> 5E59	>100b	2.9b	2.1b	2.4a	<1.0b	<1.0c	7.7a	<1.0a
<i>P.f.</i> 6E103	>100b	1.5a	1.7a	>100b	<1.0b	<1.0c	4.9b	<1.0a
<i>P.f.</i> E6-46	>100b	1.7a	1.7a	2.4a	<1.0b	6.8a	7.0a	<1.0a
<i>P.f.</i> 5C12	1.4a	2.1b	2.1b	2.2a	5.1c	6.9b	7.6a	<1.0a
<i>P.f.</i> 5C4	1.5a	1.5a	1.5a	2.0a	6.0b	6.8b	8.3a	<1.0a

^aCFU: Colony Forming Units.

^bWithin each column, means followed by different letters are significantly different at $P \leq 0.05$.

Temperature is one of the most important factors influencing the survival and efficacy of many biocontrol agents (Ghorbani et al., 2005). It should be noted that *P.f.* D7 was isolated from cool season plants and that these microorganisms generally proliferate during autumn and winter months, which coincides with the growth of downy brome. Temperature may affect the bacterium, host plant or interactions between the host and the bacterium. In our study, the optimum temperature for growth of both wild type and mutants was $\leq 24^\circ\text{C}$. Biocontrol agents require certain optimum temperatures in order to grow and carry out their activities. In eastern Washington State, for example, the optimum temperature during late fall, winter and spring is below 24°C. For bacteria, temperature influences germination, infection, latent period, lesion development, sporulation, dispersal and survival (Ghorbani et al., 2005; Agrios, 1997). These authors have suggested that to achieve the best results in the biological control of weeds, the optimum temperature should be studied for specific weed-control-agent interactions within the local habitat conditions. Our studies demonstrated that by maintaining the optimum temperature of $\leq 24^\circ\text{C}$, the growth of the bacterium could be maintained at a relatively high population for more than 48 h. These studies are necessary to develop strains that can grow and maintain high populations in temperatures that may be different from their laboratory optimum.

Population response to water potential was carried out in flasks containing PMS broth amended with 29.2 g L⁻¹ of NaCl or 200 g L⁻¹ of PEG, to obtain osmotic potentials of -2.4 MPa by two different methods (Harris, 1981). Growth of the wild type D7 in NaCl or PEG was less (8.8, 7.5 log₁₀ CFU mL⁻¹, respectively) than the CFU

from control broth ($9.5 \log_{10}$ CFU mL⁻¹) after 48 h (Table 4), and generation times were almost two times longer for *P.f.* D7 grown in both NaCl and PEG compared with the control broth. Growth of *P.f.* D7rif was similar for both NaCl and the control; however, *P.f.* D7rif did not survive 48 h in PMS amended with PEG (Table 4). The Pol⁻ variants had lower populations at 48 h for the two osmotic treatments when compared with the control and to the wild type. Compared with *P.f.* D7, growth of the Tox⁻ mutants was less after 48 h, but was similar between the control and NaCl, except for E6-46. Mutant growth decreased with PEG. Generation time was increased with PEG for the Tox⁻ isolates, and even more so with NaCl compared with the control (Table 4). Mutants grown in the NaCl broth had generation times from 2.7 h to 6.0 h, whereas mutants grown in PEG had generation times of 2.2–3.9 h. Loss of osmo-tolerance may reflect alteration of one or more physiological attributes, e.g., the reduced accumulation of osmoprotectants such as glutamate (Sugiura and Kisumi, 1985) or glycerol (Andre et al., 1988), or the maintenance of high K : Na ion ratios in the cytosol (Andre et al., 1988). The possibility of such features differing among D7 mutants warrants further attention.

Table 4 Generation time and cell numbers of batch-cultured *Pseudomonas fluorescens* D7, *P.f.* D7rif, and strains E669 (Tox⁺), 5C57 (Pol⁺), 5E59 (Tox⁻), 6E103 (Tox⁻), E6-46 (Tox⁻), 5C12 (Pol⁻), and 5C4 (Pol⁻) incubated at 22°C in PMS broth amended with NaCl or polyethylene glycol (PEG)

<i>D7 variant</i>	<i>Amendment</i>					
	<i>Generation time (h)</i>			<i>Log CFU^a mL⁻¹ at 48 h</i>		
	<i>Control</i>	<i>NaCl</i>	<i>PEG</i>	<i>Control</i>	<i>NaCl</i>	<i>PEG</i>
<i>P.f.</i> D7	1.5a ^b	2.8a	2.7a	9.5a	8.8a	7.5b
<i>P.f.</i> D7rif	1.7a	2.7a	2.9a	9.2a	9.1a	<1.0e
<i>P.f.</i> E669	1.5a	2.8a	2.7a	9.5a	8.8a	7.5b
<i>P.f.</i> 5C57	1.5a	2.7a	2.7a	9.0a	9.2a	9.0a
<i>P.f.</i> 5E59	3.4b	4.4c	3.9b	6.0b	6.6b	3.3d
<i>P.f.</i> 6E103	3.2b	6.0d	3.9b	6.2b	6.6b	2.5d
<i>P.f.</i> E6-46	1.8a	3.0a	3.0a	8.5a	7.8b	4.8c
<i>P.f.</i> 5C12	2.0a	3.8bc	2.4a	8.8a	5.4c	6.6b
<i>P.f.</i> 5C4	1.5a	3.6b	2.2a	8.9a	5.3c	6.7b

^aCFU: Colony Forming Units.

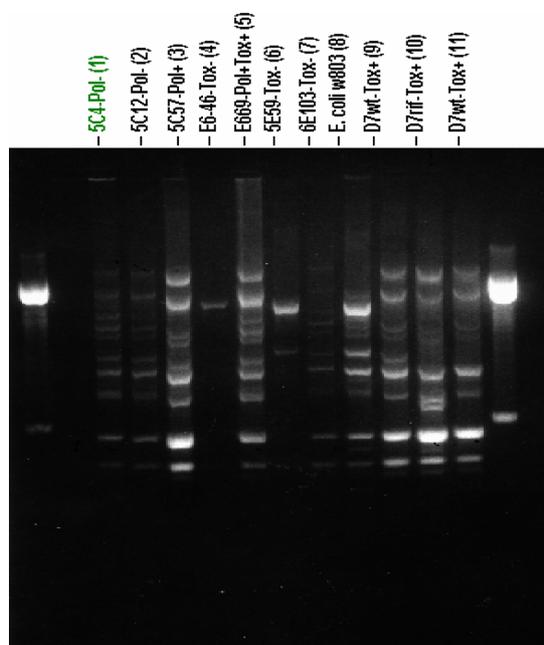
^bWithin each column, means followed by different letters are significantly different at $P \leq 0.05$.

3.2 DNA analysis of D7 toxin characteristics

The development of a successful biocontrol agent requires a thorough understanding of the ecology and physiology of the potential agent and its interaction with the environment. This may be the first step in assessing the potential effectiveness of the biocontrol agent, in addition to the physical, chemical and the genetic characteristics of the target weed. Southern blot analysis of the restriction endonuclease *EcoRI* digests of the different strains demonstrated unique Tn5 insertion sites in the genome of all the

mutants and *P.f.* D7 (data not shown). Analysis of the strains with RAPD assay showed unique patterns based on phenotypic characteristics (Figure 1). Lanes 1–11 described the different bacterial strains and their phenotypes. Analysis of the bacterial strains on agarose gel was carried out with the Image Master ID to create a database set for all the strains. The bands were found in different combinations of fingerprinting patterns. Most strains shared a large portion of the band set, while a few had unique bands as indicated by the banding patterns in Figure 1. The mean fingerprinting pattern varied between 2 bands in lane 4 (E6-46-Tox⁻) and 16 bands in lane 5 (E669-Pol⁺ Tox⁺). One band observed at about 400 bp was present in all the samples, although not readily visible in some samples.

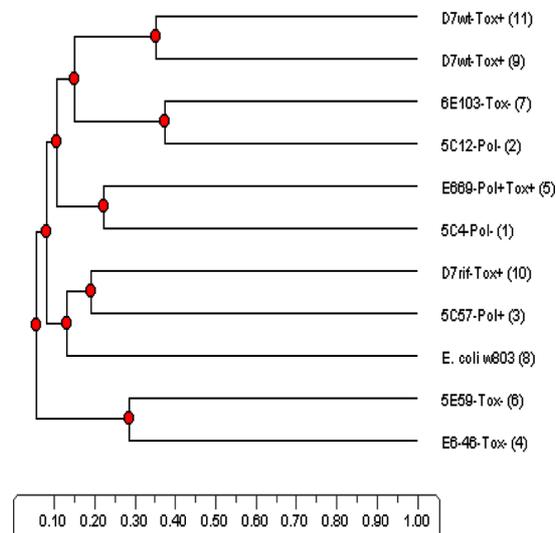
Figure 1 RAPD–PCR profiles of *Pseudomonas fluorescens* D7, *P.f.* D7rif, and strains E669 (Tox⁺), 5E59 (Tox⁻), 6E103 (Tox⁻), E6-46 (Tox⁻), 5C12 (Pol⁻), 5C4 (Pol⁻) and 5C57 (Pol⁺) generated by RAPD primers to differentiate strains with different phenotypic characteristics (see online version for colours)



Differences in the fingerprinting pattern of the strains were addressed by cluster analysis using UPGMA algorithm (Figure 2). Cluster analysis showed the wild type and the Tox⁺ Tn5 mutant (E669) clustering together. The D7rif strain and 5C57 grouped together but were dissimilar to the wild type group. The Pol⁻ strains were similar to each other. The Tox⁻ mutants were distributed throughout the clusters. These results showed that some Tn5 mutants are genotypically distinct from one another and that there are several genes involved in toxin production based on banding pattern. Our results agree with those of Kennedy et al. (1992) that suggested toxin production from *Pseudomonas* sp. strain RC1 was a result of several gene loci. Xu and Gross (1988) using Southern blot also showed that several genes may be involved in syringomycin production in *Pseudomonas syringae* pv. *syringae*. The molecular mechanism of defense by related rhizobacteria on the wheat root disease take-all has been reported to be controlled by at least three levels

of regulation of genes biosynthesis: production of cellular metabolism, global regulation that ties antibiotic production to cellular metabolism and regulatory loci linked to genes for pathway enzymes (Cook et al., 1995). Also, it has been reported that the active compounds from *P.f.* D7 inhibited the plant pathogenic fungus *Gaeumannomyces graminis* var. *tritici* (Gurusiddaiah et al., 1994). In this study, the degree of polymorphism found with RAPD seems to agree with the phenotypic characteristics of the different strains used in this study. It therefore suggests a degree of gene clustering based on phenotypic characteristics and linkages based on regulation of toxin production. The level of regulation seems to involve environmental sensing, and a secondary level that ties antibiotic biosynthesis into other metabolic processes (Cook et al., 1995; Mazzola et al., 1992).

Figure 2 Cluster analysis of the banding patterns of *Pseudomonas fluorescens* D7, *P.f.* D7rif, and strains E669 (Tox⁺), 5E59 (Tox⁻), 6E103 (Tox⁻), E6-46 (Tox⁻), 5C12 (Pol⁻), 5C4 (Pol⁻) and 5C57 (Pol⁺) using UPGMA algorithm (see online version for colours)



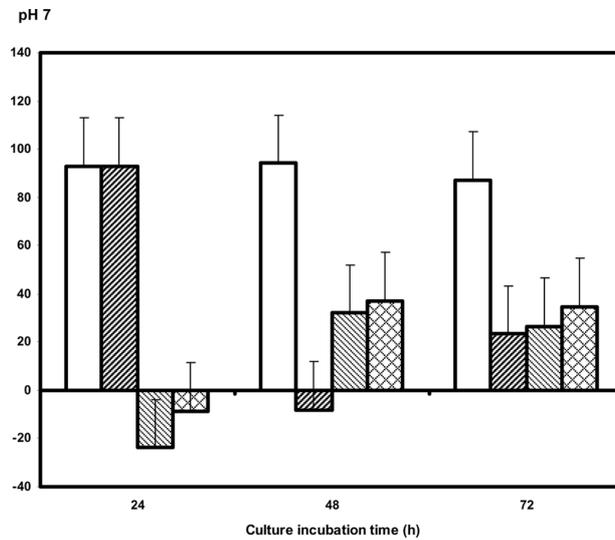
3.3 Effect of growth environment on inhibitory activity

The effect of temperature, pH and water potential on cultures used to suppress downy brome root growth was examined in agar plate bioassays using *P.f.* D7, *P.f.* D7rif and Tox⁻ mutants 5E59 and 6E103. In agar plate bioassays, supernatant of *P.f.* D7 consistently reduced root lengths of downy brome by an average of 87%, compared with less than 15% inhibition by *P.f.* D7rif and the Tox⁻ mutants. The Pol⁺ and Pol⁻ isolates were not different from the wild type in any of the bioassays (data not shown).

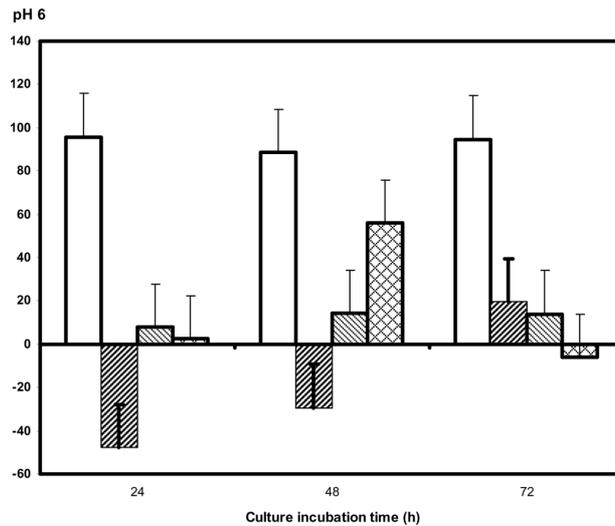
The effects of pH on culture for root inhibition studies of downy brome were studied at pH 5, 6, and 7 during a 72 h experimental period (Figure 3(a)–(c)). *P.f.* D7 inhibited root growth between 85 and 95% at the three pH levels used in this study. *P.f.* D7rif was non-inhibitory at pH 5 and 6, but showed inhibition similar to *P.f.* D7 at pH 7, 24 h. Inhibition by *P.f.* D7rif dropped off after 24 h, however. The mutants were inconsistent at each pH, and the greatest root inhibition was 56% for 6E103 at pH 6 at 48th hour. Our data suggest that *P.f.* D7 grown at the three pH levels was effective at inhibiting root

growth and development of downy brome. It also confirms that the Tox^- mutants were not effective or consistent in inhibiting root growth. These results are not surprising, considering the differences in culture growth shown in Table 2; however, it is unclear why *P.f.* D7rif was not more effective in inhibiting downy brome. Soil acidity may affect soil borne pathogens primarily through its effects on enzymes that control metabolic processes within the organism (Ghorbani et al., 2005). In eastern Washington State, most of the soils have pH values of 5.8–7.0; therefore, the effectiveness of this rhizobacterium was tested based on the environmental conditions in the area.

Figure 3 Agar plate bioassays of downy brome roots exposed to cell-free culture filtrate of *Pseudomonas fluorescens* D7, *P.f.* D7rif, and Tox^- isolates 5E59 and 6E103 grown at (a) pH 5; (b) pH 6 and (c) pH 7

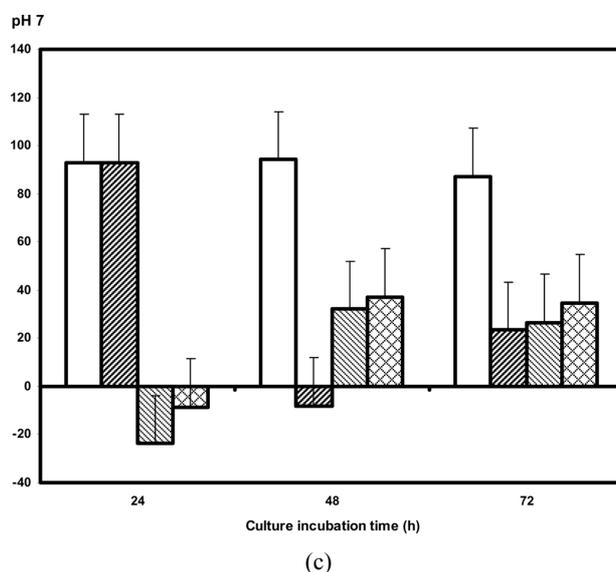


(a)



(b)

Figure 3 Agar plate bioassays of downy brome roots exposed to cell-free culture filtrate of *Pseudomonas fluorescens* D7, *P.f.* D7rif, and Tox^- isolates 5E59 and 6E103 grown at (a) pH 5; (b) pH 6 and (c) pH 7 (continued)



The wild type showed 95% root inhibition when cultures were grown at 4°C, 15°C, and 24°C, whereas *P.f.* D7rif and the Tox^- mutants showed 40% or less inhibition at any of the growth temperatures (Figure 4(a)–(c)). At 37°C the rate of root inhibition for *P.f.* D7 was significantly reduced to 50% or less; however, the percent inhibition from the mutants at 72 h was slightly higher than at other temperatures (Figure 4(d)). Our study is in agreement with the study of Mazzola et al. (1995) where a related rhizobacterium was tested to control the wheat root disease ‘take-all’ caused by the fungus *Gaeumannomyces graminis* var. *tritici*. The bacteria used were phenazine deficient (Phz^-) mutants produced by Tn5 mutagenesis, and they failed to inhibit *Gaeumannomyces graminis* var. *tritici* *in vitro*, and were similarly reduced in their ability to suppress take-all on wheat plants *in situ*.

Mutants were restored to Phz^+ by complementation with homologous DNA from a genomic library of the wild type, and the complemented strains were also fully restored for the ability both to inhibit *Gaeumannomyces graminis* var. *tritici* *in vitro* and to suppress take-all *in situ*. In another study (Kennedy et al., 2001), root inhibition by *P.f.* D7 was about 97% for *Bromus* spp. and seed germination was reduced by 38–90% compared with the controls. The authors noted also that the supernatant of *P.f.* D7 reduced the root lengths of non-*Bromus* grasses in the agar bioassay by 10–86%. In this study, *P.f.* D7 greatly inhibited root growth at temperatures between 4°C and 24°C. Bolton and Elliott (1989) showed that toxin production by a wheat-inhibitory pseudomonad was greatest at 15°C, within the temperature range of our study; however, our bioassay was conducted in agar, which is a matrix quite different from soil and lacks the competition from indigenous bacteria. Kennedy et al. (2001) showed that root

inhibition in the agar bioassay was always greater than in soil, because the agar bioassay only tested for production of plant-suppressive compounds and not the competitive or colonising ability of a bacterium in the rhizosphere.

Figure 4 Agar plate bioassays of downy brome roots exposed to cell-free culture filtrate of *Pseudomonas fluorescens* D7, *P.f.* D7rif, and Tox⁻ isolates 5E59 and 6E103 grown at (a) 4°C; (b) 15°C; (c) 24°C and (d) 37°C

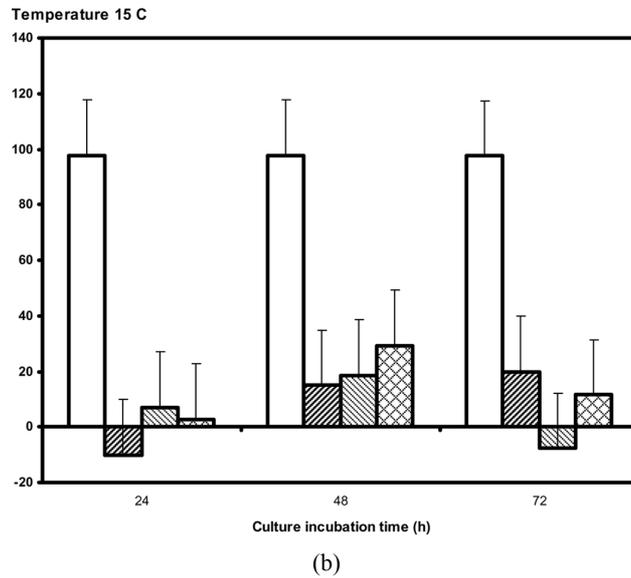
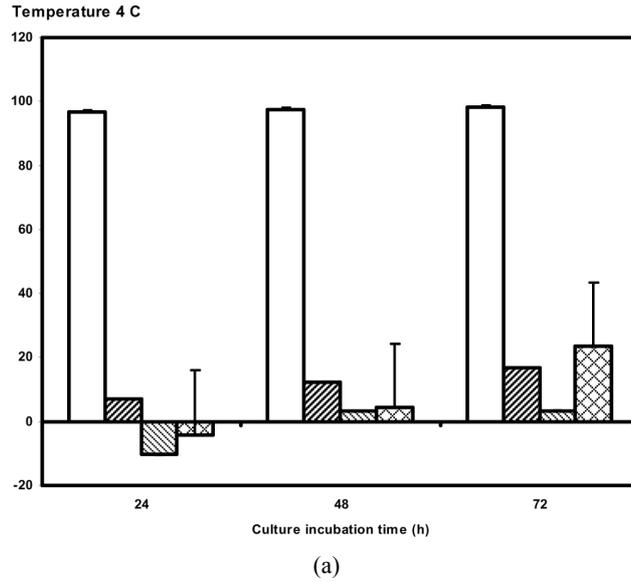
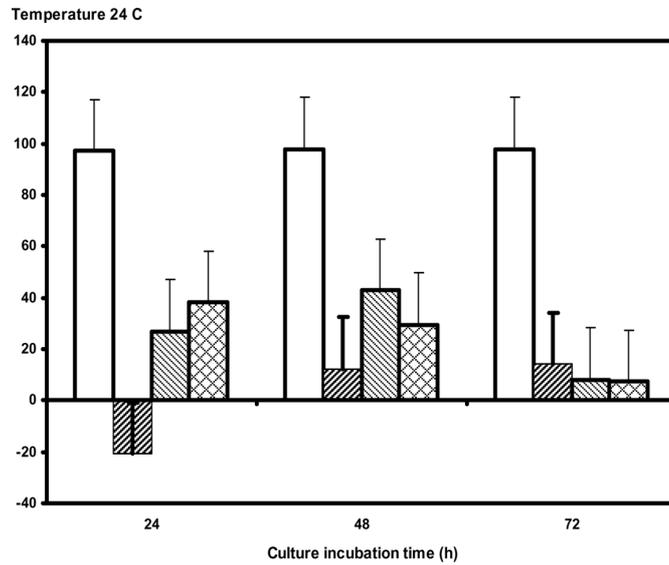
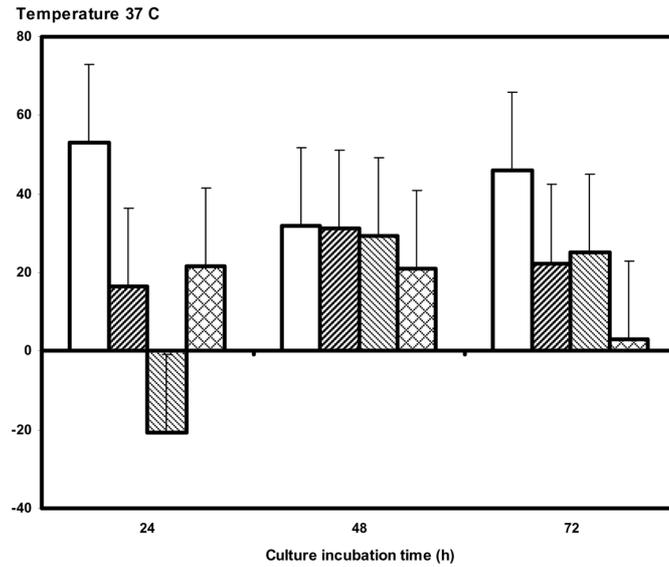


Figure 4 Agar plate bioassays of downy brome roots exposed to cell-free culture filtrate of *Pseudomonas fluorescens* D7, *P.f.* D7rif, and Tox⁻ isolates 5E59 and 6E103 grown at (a) 4°C; (b) 15°C; (c) 24°C and (d) 37°C (continued)



(c)

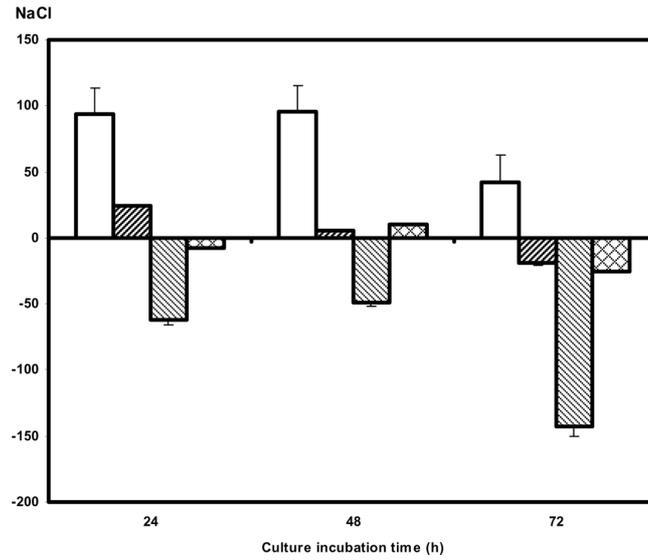


(d)

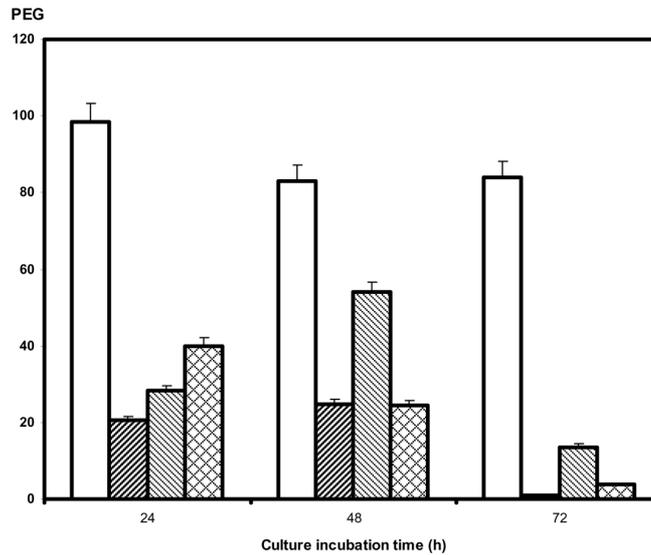
Under PMS + NaCl stress, root inhibition was between 42% and 95% for *P.f.* D7 (Figure 5(a)). *P.f.* D7rif showed only 24% inhibition at 24 h, and in most cases the mutants did not have any inhibitory effects on root growth (Figure 5(a)). Percent root inhibition was between 80% and 98% by *P.f.* D7, 1–25% for *P.f.* D7rif, and between 5% and 50% for the mutants when the cultures were grown under PMS + PEG stress (Figure 5(b)). The imposed osmotic stress by these two different methods did not reduce

the efficacy of *P.f.* D7, except for culture grown under the influence of NaCl at 72 h (Table 4). The Tox^- mutants were not effective at reducing downy brome growth with the imposed stress. Downy brome inhibition under drought conditions would more likely occur with the wild type but not the mutants.

Figure 5 Agar plate bioassays of downy brome roots exposed to cell-free culture filtrate of *Pseudomonas fluorescens* D7, *P.f.* D7rif, and Tox^- isolates 5E59 and 6E103 grown under the influence of (a) NaCl and (b) PEG



(a)



(b)

4 Conclusions and recommendations

Our data showed agreement between genetic clusters and phenotypic characteristics. Temperature, pH and water potential did not have any significant effect on the action of the wild type during the bioassay studies, except at the highest temperature (37°C) and with NaCl at 72 h. This may be one of the factors responsible for the consistent nature, which leads to an increase in inhibitory effects of *P.f.* D7 over other strains. Therefore, understanding the genetic characteristics of the bacterium producing the toxin will help in the ecological analysis of this bacterium, possible screening of weed inhibitory activity and development of successful biological weed-control systems with *P.f.* D7.

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