Colonization and survival of *Phoma macrostoma*, a weed biocontrol fungus

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Canada thistle, dandelion, chickweed, and scentless chamomile are common broadleaf weed pests of economic importance in western Canada. The fungus *Phoma macrostoma* demonstrates bioherbicidal activity and is being developed for control of these weed pests. However, little is known about the fate of the fungus in soil and plants. The aim of this research was to monitor the colonization of plant tissues by the fungus, and its movement and persistence in soil environments using a molecular detection method.

Materials and Methods

Fungal cultures and inoculum preparation. Three biocontrol isolates of *P. macrostoma* (94-44B, 85-24B, and 97-12B) were used in the studies. The isolates were maintained at -80?C in a vial of 1:1 parts of 10% skim milk to 40% glycerol. Cultures were started by thawing a vial to room temperature and spreading the contents on V-8 juice agar plates and then grown under a 16 hr photoperiod at room temperature for 2 weeks. These cultures were used to prepare a granular inoculum of the fungus using a proprietary procedure.

Field trials. Four field trials were conducted at the experimental farm of Agriculture & Agri-Food Canada, Saskatoon during 2001-2002 for monitoring the biocontrol fungus by PCR. The trials were as follows: (1) Trial 1 applied inoculum of 94-44B to soil at dosages ranging from 0 to 1000 g/m^2, (2) Trial 2 applied inoculum of 94-44B, 85-24B, and 97-12B at a fixed rate of 500 g/m^2 to the soil, (3) Trial 3 applied 85-24B to soil in 2001 at the rate of 1000 g/m^2 and monitored the fungus until 2002, and (4) Trial 4 applied 85-24B to soil at 250 and 500 g/m^2 and monitored the fungus over the growing season and at sites away from the site of application.

A randomized complete block design was used for all experiments with 2-4 replications for each treatment. The turfgrass "Overseeding Mixture" (2.84 g), dandelion seeds (0.3 g), and the fungal inoculum were broadcast on the surface of each plot and lightly hand raked in two directions. Samples of plant roots, upper-core soil samples (top 0-8 cm), lower-core soil samples (top 9-16 cm) and outside-plot soil samples (30 cm and 60 cm apart from the plot edge, respectively) were collected at designated time points for PCR (polymerase chain reaction) monitoring.

Genomic DNA extraction. Fungal cultures were grown on V-8 juice for DNA isolation using QIAGEN DNeasy? Plant Mini Kit. Plant DNA was extracted basing on Edwards et al. (1991). The UltraClean? Soil DNA Kit (MO BIO Laboratories, Inc.) was used for extraction of DNA (free of humic acid inhibitors) from soil samples.

PCR primer design and conditions. A biocontrol isolate–specific probe was generated by cloning the 1-2 kb *SacI-KpnI* fragments of the fungal genomic DNA into plasmid *pBluscript KSII*. The specific *SacI-KpnI* insert was confirmed by Southern blot and then

sequenced. A set of primers yielding a product of 853-bp was designed for PCR monitoring of the isolates. The reaction mixture consisted of 1.5U AmpliTaq Gold polymerase, 1x GeneAmp? PCR Buffer II (Applied Biosystems), 2.0mM $MgCl_2$, 0.16mM each of dNTP, 0.25?M of each primer, and 10ng template DNA. The PCR program was 94?C for 10 min; 35 cycles of 94?C 2 min, 60?C 2 min, 72?C 3 min; 72?C for 10 min; and hold at 4?C.

Results

Colonization of P. macrostoma in plants

In all of the field trials, colonization of plants by the biocontrol fungus was detected in 69.2% of the total root samples collected from treated plots starting at the date of application and up to two months post application. In contrast, it was only occasionally (5.9%) and weakly detected in the untreated control samples. Based on Trials 1 and 2, the frequency of positive detection did not decrease significantly by comparing a frequency of 66.7% in the two-month samples with 60.0% in the four-month samples. The fungus was not detected in the root samples one year after treatment.

Colonization of P. macrostoma in soil

In the field trial with 94-44B (Trial 1), the presence of DNA of the biocontrol fungus was monitored in upper core soil samples taken approximately 2 and 4 months post treatment. In both cases, the fungus was detected in the treated core soil, but not in the untreated control. (Table 1).

Trial 2 showed that the three different isolates of the fungus were detected in treated soil cores (Table 2). No significant differences between these isolates were found.

For soil samples taken within the first two months post treatment, the biocontrol fungus was detected in 95.7% of the total treated soil samples, while in only 1.9% in the samples taken from the untreated control and plot edges. At 4 months post treatment, the bands obtained by PCR analysis became much weaker, and the frequency of positive detection in the upper core soil samples decreased from 93.3% to 80.0%, based on data in Trials 1 and 2. This result indicated that the biocontrol fungus was still present in soil, but at lower levels than seen at 2 months after its application.

Movement of P. macrostoma in Soil

The biocontrol fungus showed itself as a low mobility microorganism. It was not detected in the 4-month soil samples taken at 30-60cm away from the plot edge (Tables 1-2). Meantime, we did not detect the fungus from lower core soil samples as well, suggesting that it was unable to move downward, beyond the depth of placement, i.e. no more than 8cm (Table 4).

Persistence of P.macrostoma in soil

The biocontrol fungus was detectable up to 4 months post treatment, however, it was undetectable one year later (Tables 1-3, 5). This implied that the fungus survived for several months but did not last for more than one year under natural soil conditions.

Discussion

The research has shown that the biocontrol fungus colonizes plants when they come into contact with the fungus by an inundative application to the soil. However, the colonization of plants by the fungus had a tendency to decline with time. This is a

positive feature in a microbial product because the fungus will not multiply and increase on hosts in the environment.

The biocontrol fungus will likely have minimal impact on the environment. The effects of the fungus were mostly limited to the site of placement and in the season of application. The fungus was not able to move any significant distance in soil unassisted and its low mobility was demonstrated by its absence outside the treated plots and at lower soil depths. The fungus persisted in the soil for several months, but the number of viable propagules significantly declined during this time.

Therefore, in the longer term, this biocontrol fungus is unlikely to persist in the soil. The fungus may be easily observed at the time of application and up to 30 days after, but there is no carryover to the following year. Related field research has also shown that susceptible crops may be sown into soils previously treated with the biocontrol fungus and do not show any signs of chlorosis or effects on emergence and plant growth.

Table 1. Detection of biocontrol fungal DNA in plant roots and soil samples taken from within the plots and from plots edges of Trial 1.

Dose g/m^2	Rep	Root		Core soil		30cm soil	60cm soil
		2 mo.	4 mo.	2 mo.	4 mo.	4 mo.	4 mo.
1000	I	+	-	+	+	-	-
	III	+	?	+	+	/	/
	IV	+	+	+	+	-	-
250	I	-	?	+	-	-	-
	III	-	-	+	+	/	/
	IV	-	?	+	+	-	-
63	I	+	?	+	-	-	-
	III	-	-	+	+	/	/
	IV	+	?	-	+	-	-
0	I	-	-	-	-	-	-
	III	-	-	-	-	/	/
	IV	-	?	-	-	-	-

* + = DNA detected; - = DNA not detected; ? = DNA weakly detected; /=not applicable.

Table 2. Detection of biocontrol fungal DNA in plant roots and soil samples taken from within the plots and from plots edges of Trial 2.

Isolate	Rep.	Root		Core soil		30 cm soil	60 cm soil
		2 mo.	4 mo.	2 mo.	4 mo.	4 mo.	4 mo.
Control	I	-	-	-	-	-	-
	IV	-	-	-	-	-	-
94-44B	I	+	-	+	+	-	-
	IV	+	?	+	-	-	-
85-24B	I	+	?	+	+	-	-
	IV	-	-	+	+	-	-
97-12B	I	+	?	+	+	-	-
	IV	+	-	+	+	-	-

Table 3. Detection of biocontrol fungus (85-24B) DNA in plant roots and soil samples taken one year post treatment from within the plots and from plots edges of Trial 3.

Treatment	Root			Core soil			30cm soil		60cm soil	
	I	III	IV	I	III	IV	I	IV	I	IV
1000 g/m^2	-	-	-	-	-	-	-	-	-	-
0 g/m^2	-	-	-	-	-	-	-	-	-	-

Table 4. Detection of biocontrol fungal DNA in plant roots and soil samples taken from within the plots and from plots edges of Trial 4 -- Spatial monitoring at 5wk

Treatment	Rep.	Plant roots	Soil samples			
			Upper core	Lower core	30cm apart	60cm apart
Control	I	-	-	-	-	-
	II	-	-	-	/	/
85-24B	I	-	+	-	-	-
250 g/m^2	II	-	-	-	/	/
85-24B	I	-	+	-	-	-
500 g/m^2	II	+	+	-	/	/

Table 5. Detection of biocontrol fungal DNA in plant roots and core soil samples taken from within the plots and from plots edges of Trial 4—Temporal monitoring.

Treatment	Type	Rep.	0wk	1wk	2wk	3wk	5wk	7wk	9wk
Control	root	I	/	/	-	-	-	-	-
		II	/	/	-	-	-	-	-
	core	I	-	-	-	-	-	-	-
		II	-	-	-	-	-	-	-
85-24B	root	I	/	/	+	+	-	+	-
250 g/m^2		II	/	/	+	-	-	-	+
	core	I	+	+	+	?	+	+	+
		II	+	+	+	?	-	+	+
85-24B	root	I	/	/	+	+	-	+	+
500 g/m^2		II	/	/	+	+	+	+	-
	core	I	+	+	+	+	+	+	+
		II	+	+	+	+	+	+	+

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