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Efficacy of *Phoma macrostoma*, a Bioherbicide, for Control of Dandelion (*Taraxacum officinale*) Following Simulated Rainfall Conditions

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ABSTRACT

This study evaluated the efficacy of the bioherbicide *P. macrostoma* under simulated rainfall conditions to determine whether bioactivity was retained in the soil and whether bioherbicidal metabolites would be soluble in water possibly posing a risk to off-site hosts. Various application rates of the bioherbicide were leached with water equivalent to 25-250 mm of precipitation and tested for efficacy before and after leaching using a dandelion bioassay. The percolated water was collected in six fractions (F1-F6) and similarly tested for bioactivity using a dandelion bioassay. Relative concentrations of macrocidin A, the main bioherbicidal metabolite, were estimated by HPLC. DNA-specific primers were used to detect the presence of the fungus in soil. Of three soil types treated with the bioherbicide, all lost the ability to control dandelion after being leached. Clay, as compared to sandy loam and greenhouse soil mix, retained the most bioherbicidal activity. The bioherbicidal metabolite was soluble in water with up to 80% macrocidin A being released in 75 mm of water and bioactivity occurring in fractions F1-F3, with much less or no activity in fractions F4-F6. The impact of water on the distribution of the living component of the bioherbicide was not clearly determined. The study shows that water releases macrocidins from the bioherbicide allowing the compound to be taken up by the roots of the plant, subsequently resulting in plant death. When soils are at field capacity or drier, this amount of rainfall is of little concern as the bioactivity is localized. However under saturated soils, macrocidins may be released in the soil water and if not taken up by the plants, run-off water may pose a risk to off-site hosts. Factors that mitigate the risks associated with off-site movement are discussed.

Keywords: biological control, broadleaved weeds, environmental fate
Abbreviations: EP, end product; **HPLC**, high performance liquid chromatography; **SE**, standard error; **TGAI**, technical grade active ingredient

INTRODUCTION

The coelomycete fungus *Phoma macrostoma* Mont. is a ubiquitous organism with a cosmopolitan distribution and wide host range (Farr *et al.* 1989). In nature, the fungus occurs mostly on woody hosts, especially members of the *Rosaceae*, where it behaves as a weak parasite or wound pathogen, and is rarely considered a plant pathogen of significance (Boerema and Dorenbosch 1970; Boerema *et al.* 2004). However, isolates of *P. macrostoma* derived from Canada thistle (*Cirsium arvense* L. (Scop.)) have been shown to exhibit bioherbicidal properties against Canada thistle, dandelion (*Taraxacum officinale* Weber ex F.H. Wigg.) and other economically important broadleaved weeds (Bailey and Derby 2001). When *P. macrostoma* is applied in a granular formulation to the soil, photobleaching of the leaves and root growth inhibition is observed in susceptible hosts like Canada thistle and dandelion. Activity is attributed to phytotoxic fungal metabolites known as macrocidins (Graupner *et al.* 2003).

Phoma macrostoma is efficacious as a bioherbicide providing 67-94% dandelion control under the semi-arid growing conditions typical in Saskatchewan (Zhou *et al.* 2004). Under these conditions, the risk of non-target infection by *P. macrostoma* was shown to be minimal due to the biology of the organism (i.e. rain-splash dispersal of conidia, lack of sexual stage, and saprophytic nature), its limited mobility and dispersion in soil, and poor persistence from year to year.

On the Canadian prairies, rainfall is often a limiting factor for the growth of plants and other living organisms. Historically in Saskatoon, Saskatchewan, the monthly precipitation averaged from 1971-2000 was 49 mm in May, 61 mm in June, 60 mm in July, and 39 mm in August (Available online at www.weatheroffice.gc.ca). However, daily extremes in precipitation equivalent to 59 mm on May 18, 1999; 97 mm on June 24, 1983; 72 mm on July 30, 1904; and 84 mm on August 3, 1945 have also been recorded. Other parts of Canada, such as the coastal environments, may experience higher levels of precipitation. Therefore, depending on the frequency and intensity of precipitation, bioherbicides may be subject to leaching or run-off that both reduces efficacy and increases the risk of exposure to nontarget organisms.

Prior to testing this bioherbicide in other environments across Canada, laboratory and greenhouse studies were conducted simulating varying levels of precipitation. Studies were designed to determine whether bioherbicide efficacy is retained in the soil at the site of application or if precipitation facilitates off-site movement of the bioherbicide thereby posing a risk to nontarget plants.

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MATERIALS AND METHODS

Experiments were designed to pass water through the technical grade active ingredient (TGAI) of *P. macrostoma* such that a bioassay could then be used to evaluate the efficacy remaining in the solid portion of the TGAI and that which could be attained from the leachate water. Initial experiments were conducted using a soil-free system which was not encumbered by various soil properties that could potentially bind or interfere with movement of the bioherbicide. However, subsequent experiments were conducted using a range of soils including clay, sandy loam, and a greenhouse soil mix to simulate more natural environments and to investigate the role of soil organic matter. The TGAI used in all experiments was characterized as having 1×10^6 particles g^{-1} ranging in size from 425-1200 µm having 75% particle viability and containing 250 macrocidin units g^{-1}.

Dandelion bioassay for measuring efficacy

A dandelion bioassay was used to determine the efficacy of the TGAI and the leached water fractions in soil and soil-free systems. In the soil system, pots containing different soils that had been treated with the TGAI and then leached with water were used as source pots and sown with 40 dandelion seeds for the bioassay. With the soil-free system, the TGAI was scraped from the filter paper following leaching and then air dried. The scraped TGAI was then applied to source pots containing 40 dandelion seeds by broadcasting the TGAI on the surface of 10 cm^2 pots filled to ¾ capacity with soil-less mix [(one part sand to 12 parts of a 1: 2 sphagnum peat moss: vermiculite mixture amended with 1% (w/v) of 16: 8: 12 (N: P_2O_5: K_2O) and 0.2% of 0:20:0 (N:P:K) controlled-release fertilizers (The Scott's Company, Marysville, Ohio)].

Source pots were placed in the greenhouse with a diurnal regime comprising a 16 h photoperiod provided by artificial light from high-pressure sodium lamps (230 µE m^{-2} s^{-1}) and temperatures ranging from a daytime high of 20°C to an overnight low of 15°C. Source pots were loosely covered with white row cover fabric for seven days to prevent drying while seedlings emerged after which the fabric was removed. Pots were watered daily using a low-impact misting nozzle. Bioherbicidal activity was determined by recording the total number of dandelions per pot at 21 days after seeding. Efficacy was expressed as the percentage weed control according to the formula: % Weed Control = (1– (Number of Treated Plants/Number of Untreated Plants)) × 100.

The leached water fractions were evaluated for bioherbicidal activity by separately applying the collected water fractions to 10 cm^2 pots filled to ¾ capacity with soil-less mix and sown with 40 dandelion seeds; these are known henceforth as the leachate pots. No TGAI was present in the leachate pots. Water fractions were applied slowly, percolating via gravity until all the liquid was absorbed by the soil in the pot. The leachate pots were placed in the greenhouse and rated for % dandelion control after 21 days as described above.

Soil-free system

1. Efficacy of TGAI and leachate

Moistened 9 cm diameter Whatman #1 filter paper in a Buchner funnel was inoculated with *P. macrostoma* at rates equivalent to 0, 16 and 64 g m^{-2} and leached under vacuum pressure with four 250 ml aliquots of water. Each fraction (F1-F4) was collected separately. Based on the diameter of the filter paper and funnel, the total 1 L water volume applied was equivalent to 128 mm of precipitation. Each leachate fraction (F1-F4) was applied individually to pots sown with dandelion seed as described in the bioassay.

The leached filter paper was then air-dried and the remaining TGAI was scraped from the surface of the filter paper for broadcasting on the source pots sown with dandelion seed as previously described for dandelion bioassays. To test for residue remaining on the filter paper, scraped filter papers were then placed in glass Petrie plates, moistened with water, and then broadcast with 40 dandelion seeds. Each plate was sealed with plastic wrap to prevent moisture loss and incubated for seven days at room temperature (20 ± 3°C). Dandelion germination and photobleaching of seedlings was recorded.

2. Water solubility of macrocidins

To determine the solubility of macrocidins in water, an experiment was conducted to compare the relative amount of macrocidin A in either the TGAI or an end product (EP) formulation before and after leaching with varying amounts of water. Using the soil-free system, 9 cm diameter #1 Whatman filter papers were placed in three Buchner funnels. The TGAI or EP of *P. macrostoma* was applied to each filter paper at the rate of 256 g m^{-2}. Each funnel received one of three volumes of reverse-osmosis purified water; 195, 390, or 585 ml which were equivalent to 25, 51, and 76 mm of precipitation, respectively. Water was applied in 50 ml aliquots and was poured over the filter paper and allowed to drain via gravity for 2 min after which vacuum pressure was applied to draw through any remaining water. This process was repeated until the entire water volume had been applied to each funnel. The experiment was performed in duplicate both with the TGAI and EP (four experiments in total). After leaching the samples were placed in a flow hood to air dry for four days, then the solid portion of the samples were freeze dried and stored at -80°C until being analysed by HPLC. Reference samples of TGAI and EP that did not receive water treatments were also freeze dried and stored at -80°C.

Three 100 mg samples of the freeze dried material from each water exposure treatment and the associated reference samples were weighed. One-ml of 60% aqueous ethanol was added to each sample and the solution vortexed for 1 min, followed by centrifugation at 2000 rpm for 20 min. The supernatant was then syringe filtered (0.45 µm) into a vial for HPLC analysis which was performed on a Waters 2695 Alliance HPLC module equipped with a photo diode array detector. A Waters Symmetry C18 Column 3.0 mm × 150 mm (5 µm particle size) was used for the analysis. The eluents used for chromatography were as follows: eluent A was 0.05% trifluoroacetic acid in purified water (Millipore Super Q system, Bedford MA) while eluent B was 0.05% trifluoroacetic acid in acetonitrile (HPLC grade, Fisher, Canada). Gradient elution was used for separation with starting conditions as 95% eluent A to 5% eluent B. Over a period of 20 min, the eluent system was adjusted to 5% eluent A and 95% eluent B, followed by a 10-min hold at that ratio. Starting conditions were resumed over 5 min and then equilibrated for 10 min.

Peak areas at 280 nm for each sample were determined for macrocidin A. Comparisons of peak areas for samples exposed to each water volume against the TGAI or EP reference samples permitted calculation of the percentage reduction of macrocidin A = ((Peak area $_{\text{no water}}$ − Peak area $_{\text{water volume}}$)/Peak area $_{\text{no water}}$)). For each of the four experiments, the % reduction of macrocidin A is expressed as the mean and standard error of the three HPLC measurements each based on a 100 mg sample of TGAI or EP. The mean and standard error over the 4 trials was also calculated.

Soil systems

1. Efficacy of TGAI and leachate with a greenhouse soil mix

A preliminary experiment was conducted to evaluate leachate water that passed through greenhouse soil mix containing the TGAI of *P. macrostoma* for bioherbicidal activity. This preliminary experiment was conducted in duplicate, but the latter experiment incorporated a slightly different greenhouse soil mix. In the first trial, 12.5 cm diameter plastic pots were filled to ¾ capacity with a general greenhouse soil mix [(3 parts sandy loam: 2 parts peat: 2 parts vermiculite: 2 parts sand) with 150 g of 26-13-0 N: P: K per 75 L of ingredients], then brought to field capacity with water and inoculated with the TGAI of *P. macrostoma* at rates equivalent to 0, 16, 32 and 64 g m^{-2}. After leaching these became the 'source pots' for the dandelion bioassay. Wetting of the soil was a necessary step prior to inoculation to facilitate both the movement of leachate fractions and to ensure that the soil was compacted to prevent the development of channels where the inoculum would be washed through the soil. In the second trial, thistle greenhouse soil mix (3 parts sandy loam: 2 parts peat: 1 part vermiculite) was utilized which differed from the general green-

house soil mix by the absence of fertilizer.

Four 250 ml aliquots of water, referred to hereafter as leachate fractions F1-F4, were applied consecutively to the surfaces of the pots at each treatment rate and allowed to percolate (leach) through the soil before being recollected as separate fractions. The total application of 1 L of water to each pot was equivalent to 125 mm of precipitation or 31 mm per fraction. Unleached pots for each rate of bioherbicide (i.e. pots to which no water fractions were applied) were used as treatment controls. Both leached and unleached pots were used for the dandelion bioassay. To assess the bioherbicidal activity in the water fractions, 100 ml of each fraction was applied to leachate pots as described in the dandelion bioassay.

2. Efficacy of TGAI and leachate with three soil types

Clay, sandy loam, and the thistle greenhouse soil mix were characterized by soil testing (**Table 1**). For each soil type, 12.5 cm diameter plastic pots were filled to ¾ capacities, then brought to field capacity with water and broadcast with TGAI of *P. macrostoma* at a rate of 0 or 128 g m^{-2}. Four 500 ml fractions (F1-F4) of water were leached through the pots, individually recollected post filtration and similarly tested for bioherbicidal activity using the dandelion bioassay. Unleached source pots were used as treatment controls. The dandelion bioassay was performed with the source pots and the leachate using 250 ml of each water fraction (F1-F4).

After completion of the dandelion bioassays, the plants were removed from the source pots and discarded. Source pots were then subjected to further leaching whereby two additional 500 ml water fractions, designated F5 and F6, were applied to each pot and allowed to percolate through the soil, collecting the water fractions separately. Source pots were then tested for bioherbicidal activity using the dandelion bioassay and water fractions F5 and F6 were tested for bioherbicidal activity in leachate pots using the dandelion bioassay.

The initial 2L volume of water applied to the 12.5 cm diameter pots was equivalent to 250 mm of precipitation in total or 62.5 mm per fraction. The subsequent 1L volume of water was equivalent to 125 mm precipitation or 62.5 mm per fraction and was received about 28 days after the initial water treatment. The experiment was repeated three times.

3. Fungal DNA amplification from soil

Dandelions were removed from the source pots used in the soil system experiments and discarded. Five soil cores were taken from each pot, combined, ground to a fine powder using a mortar and pestle and 0.3 g used as starting material for DNA extractions, which were performed using the UltraClean Soil DNA Extraction Kit (MO BIO Laboratories, Solana Beach, California) according to the manufacturer's instructions. Detection limits for *P. macrostoma* were established using a range of positive controls that were created by extracting soil samples comprising bioherbicide rates equivalent to 125, 16, 4, 2, 1, 0.5, 0.25 and 0 g m^{-2}. Both source pot samples and positive control samples were subjected to PCR amplification using *P. macrostoma* bioherbicidal strain-specific primers Pm853L and Pm853R (synthesized by Invitrogen Life Technologies) according to methods published by Zhou *et al.* (2004). PCR reactions were performed in a total volume of 25 µL comprising 5 ηg of purified genomic DNA template, 0.025 µM of each primer, 160 µM of dNTP mix (New England Biolabs, Ltd., Pickering, Ontario), 2.0 mM of $MgCl_2$, 1.5 U of *Taq* DNA polymerase (Promega Corp., Madison, Wisconsin) and 1 × PCR reaction buffer (5.0 mM Tris-HCl [pH 8.0], 10.0 mM NaCl, 0.01 mM EDTA, 0.1 mM DTT, 5.0% glycerol and 0.1% Triton X-100). All PCR reactions were performed in a PTC DNA Engine™ thermocycler (MJ Research Inc., Waltham, Massachusetts) with an initial denaturation of 3 min at 94°C followed by 35 cycles of denaturetion (60 s at 94°C), annealing (60 s at 60°C) and extension (90 s at 72°C), and a final extension of 10 min at 72°C. Two successive rounds of PCR were conducted on each sample under identical conditions with 1 µL of the initial reaction product used as template DNA for the second round of amplification. PCR products were separated by electrophoresis on 2% agarose containing 1 × Tris acetate-EDTA buffer. Gels were run at 100 V for 3 h, and then stained with 1.0 µg/ml ethidium bromide for 15-20 min before being visualized and photographed under UV light. All PCR amplifications were performed in duplicate for purposes of reproducibility.

Table 1 Characteristics of the three soils used in the advanced soil system.

	Clay	Sandy loam	Greenhouse soil mix¶
% Sand	20	65	63
% Silt	36	25	52
% Clay	44	10	11
% Organic matter	3.9	4.2	8.3
% Organic carbon	2.2	2.4	4.8
pH	7.9	7.6	6.9
Cation exchange capacity meq/100 g	29.3	15.0	21.3
AAE ¶¶ cations-Ca meq/100 g	34.9	19.0	19.7
AAE ¶¶ cations-Mg meq/100 g	9.7	4.0	5.9
Available nitrate-N mg/kg	122	173	177
Available phosphate-P mg/kg	18	43	41
Available potassium mg/kg	520	595	588

¶ 3 parts sandy loam soil, 2 parts peat, 1 part vermiculite, no fertilizer
¶¶ Ammonium acetate extractable

Statistics

Experiments were conducted using a random complete random block design with four replicates and were repeated two or three times. Data were subjected to analysis of variance (SAS version 6). Trials that were not significantly different were combined. Data were presented as combined means and standard errors. Duncan's multiple range tests were used for means comparisons of treatment for % dandelion control and % reduction of macrocidin A at P=0.05.

RESULTS AND DISCUSSION

Soil-free system

1. Efficacy of TGAI and leachate

The technical grade active ingredient comprises particle sizes in the range 425 to 1200 µm. While larger particles were scraped from the filter paper after leaching, the finer particles became embedded such that the volume of TGAI recovered from the filter paper after leaching was reduced in comparison to the amount applied. Inevitably, slightly less TGAI than that originally applied to the filter paper was transferred to the source pots for the dandelion bioassay. At 16 g m^{-2}, there was a 30% reduction in the TGAI after leaching whereby mean recovery from leached samples was 0.14 g $filter^{-1}$ ± 0.01 SE compared to 0.2 g $filter^{-1}$ ± 0.0 SE in unleached samples. Similarly, at 64 g m^{-2}, there was a 25% reduction in TGAI after leaching whereby mean recovery from the leached samples was 0.6g $filter^{-1}$ ± 0.01 SE compared to 0.8 g $filter^{-1}$ ± 0.0 SE in the unleached samples.

Germination of dandelion seeds on filter paper embedded with TGAI residue (after leaching and scraping) was 72.5% ± 3.2 SE (Trial 1) and 76.9% ± 1.9 SE (Trial 2) at the 16 g m^{-2} rate of application and 80.6% ± 3.0 SE (Trial 1) and 77.5% ± 1.0 SE (Trial 2) at 64 g m^{-2}. At both application rates, 90% of the plants were photobleached. Similarly, germination of dandelion at the 0 g m^{-2} bioherbicide rate was 81.3% ± 4.3 SE, but in contrast all plants were green and healthy. Hence while germination was unaffected by the bioherbicide, the associated effect of photobleaching killed the emerging seedlings.

The TGAI scraped from the filter paper after leaching was also used in a dandelion bioassay. Pot treated with the bioherbicide contained significantly fewer dandelions than untreated pots (**Table 2**). At the lower rate of application, leached TGAI produced slightly less control of dandelion (75%) than at the higher rate (98%), although this difference between treatment rates was not significant in Trial 2. When the TGAI was not leached the level of control was

Table 2 The mean number of dandelion ± SE and % dandelion control three weeks after the application of the bioherbicide *Phoma macrostoma* at three rates of the technical grade active ingredient that had been leached through filter paper with 1L of water or not leached with water before broadcasting to the soil surface.

Treatment	Bioherbicide rate	Dandelion/pot		% Dandelion Control	
	g m^{-2}	Trial 1	Trial 2	Trial 1	Trial 2
Leached	0	22.0 ± 1.1	28.5 ± 1.4	0 a¶	0 a
	16	5.5 ± 0.5	8.0 ± 2.5	75 b	72 b
	64	0.5 ± 0.5	1.5 ± 1.0	98 c	95 bc
Not leached	0	16.8 ± 1.9	19.3 ± 3.1	0 a	0 a
	16	0.5 ± 0.5	0.8 ± 0.5	97 c	96 bc
	64	0.0 ± 0.0	0.0 ± 0.0	100 c	100 c

¶ Only the mean % dandelion control was analyzed using Duncan' multiple range test; different lower case letters within a column for represent significant differences at P=0.05

Table 3 The mean number of dandelions ± SE and % dandelion control three weeks after the application of the water fractions (F1-F4) that had been collected after leaching through three rates of the bioherbicide *Phoma macrostoma* on filter paper.

Trial	Rate	Dandelion/pot				% Dandelion Control			
	g m^{-2}	F1	F2	F3	F4	F1	F2	F3	F4
1	0	28.3 ± 0.6	27.5 ± 0.9	27.8 ± 2.6	26.3 ± 0.9	0 a¶	0 a	0 a	5 ab
	16	26.0 ± 1.8	28.3 ± 1.8	26.3 ± 2.0	27.0 ± 1.1	5 ab	0 a	5 ab	2 a
	64	0.8 ± 0.5	7.5 ± 2.9	21.8 ± 1.7	24.8 ± 1.7	97 d	73 c	21 b	10 ab
2	0	21.0 ± 0.8	21.5 ± 1.9	24.5 ± 1.7	24.5 ± 2.8	16 a	14 a	2 a	2 a
	16	12.8 ± 6.1	18.3 ± 1.8	18.0 ± 0.7	23.0 ± 1.0	49 bc	27 ab	28 ab	5 a
	64	0.3 ± 0.3	9.0 ± 2.0	10.8 ± 2.3	18.8 ± 2.0	99 d	64 c	57 c	25 ab

¶ Only the % dandelion control was analyzed using Duncan's multiple range test; different lower case letters for all fractions and rates within a trial represent significant differences at P=0.05

similar at both rates (96-100%). Therefore, the TGAI retained much of its biological activity despite receiving 128 mm precipitation. This 20-25% loss in activity at the lower rate was similar to the proportionate reduction in TGAI that was applied to the source pots after being scraped from the filter. Hence, in the soil-free system losses in efficacy may be attributed to the reduction in the quantity of bioherbicide applied and not from the leaching. Overall, biological activity was retained at the site of placement (i.e. on the filter paper).

Some leachate fractions demonstrated bioherbicidal activity, but this was dependent on the rate of application and the specific fraction (**Table 3**). At 0 g m^{-2}, there was negligible dandelion control in Trial 1 and from 2-16% control in Trial 2. This rate established a base line of control since some minor phytotoxic components resulting in mortality were present in the soil-free system in the absence of the bioherbicide. At 16 g m^{-2}, there was no bioherbicidal activity in leachate fractions F1-F4 in Trial 1. In Trial 2, fraction F1 demonstrated 49% dandelion control, but other fractions (F2-F4) demonstrated negligible levels of control which were not significantly different to the untreated controls. At 64 g m^{-2}, leachate fraction F1 demonstrated 97-99% dandelion control, but the level of control declined thereafter with each successive fraction such that by fraction F4 weed control compared to the untreated control was negligible. The leachate fractions demonstrated that a bioactive component of the bioherbicide can be released in water, albeit primarily concentrated in fractions F1 and F2. Bioactivity is not sustained as latter fractions often produce little or no weed control. In nature fraction F1 was equivalent to 32 mm rainfall which would normally be utilized by growing plants at the site of placement. Fractions F1 and F2 combined, equate to 64 mm of precipitation, which is similar to the long-term monthly average of 60 mm received in June or July in Saskatoon (Available online at www.weatheroffice.gc.ca). The long-term cumulative seasonal precipitation in Saskatoon for May-August is 209 mm which is almost double the 128 mm cumulative total for fractions F1-F4. The simulated precipitation in the soil-free system is therefore representative of a slightly drier than normal growing season for Saskatoon and as such there is little risk of the bioactive component moving off-site since this volume of water will be utilized by the plants at the site of application. The bioactivity present in F1 and F2 may ultimately improve efficacy by making the bioherbicide more available for uptake by the roots of the target weeds. By manipulating application rates the risk of nontarget effects from run-off may also be mitigated especially if extreme rainfall events occurred as less bioactive component is leached at lower application rates.

2. Water solubility of macrocidins

Macrocidins are phytotoxic metabolites produced by *P. macrostoma* (Graupner *et al.* 2003). Macrocidin A is the major component associated with the bioherbicidal activity of the fungus (Graupner *et al.* 2006). HPLC analysis showed that inoculum on filter paper leached with water lost

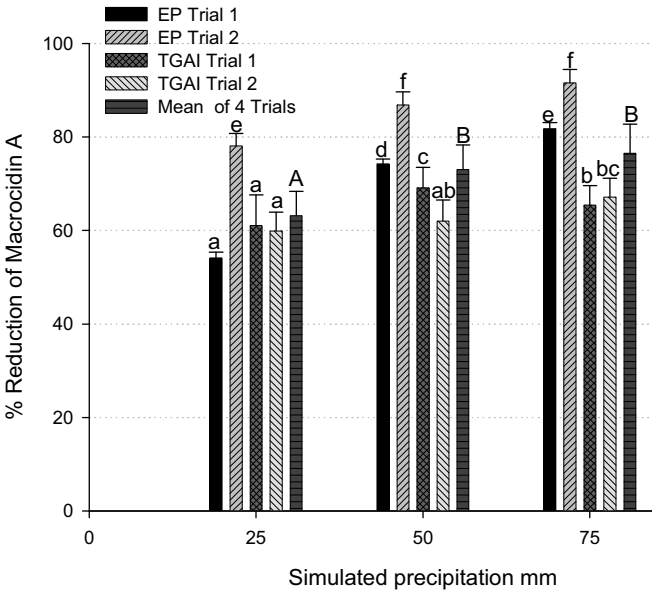

Fig. 1 The % mean reduction of macrocidin A and standard error after leaching an end product (EP) formulation and technical grade active ingredient (TGAI) of the bioherbicide *Phoma macrostoma* on filter paper in a soil-free system with 25-75 mm of water relative to a reference standard that was not leached. Lower case letters represent differences among formulation-trials and upper case letters represent differences among the water volumes using a Duncan's multiple range test at P=0.05.

Table 4 The mean number of dandelion ± SE and % dandelion control three weeks after broadcasting the technical grade active ingredient of the bioherbicide *Phoma macrostoma* at four rates to the surface of a greenhouse soil mix that had been leached with 1L of water before seeding with dandelion.

Treatment	Bioherbicide rate	Dandelion/pot		% Dandelion Control	
	g m^{-2}	Trial 1	Trial 2	Trial 1	Trial 2
Leached	0	25.0 ± 0.8	16.0 ± 1.1	0 a¶	0 a
	16	26.5 ± 1.3	13.3 ± 0.8	0 a	17 a
	32	27.5 ± 1.3	13.0 ± 2.4	0 a	19 a
	64	Not done	11.5 ± 1.9	Not done	28 a
Not leached	0	26.5 ± 2.1	14.3 ± 4.5	0 a	0 a
	16	0.5 ± 0.5	0.0 ± 0.0	98 b	100 b
	32	0.0 ± 0.0	0.0 ± 0.0	100 b	100 b
	64	Not done	0.0 ± 0.0	Not done	100 b

¶ Only % dandelion control was analyzed using Duncan' Multiple Range Test.; different lower case letters within a column represent significant differences at P=0.05

Table 5 The mean number of dandelions ± SE and % dandelion control three weeks after the application of the water fractions (F1-F4) that had been collected after leaching through greenhouse soil mix containing four rates of the bioherbicide *Phoma macrostoma*.

Trial	Rate	Dandelion/pot				% Dandelion Control			
	g m^{-2}	F1	F2	F3	F4	F1	F2	F3	F4
1	0	30.0 ± 0.7	29.0 ± 1.8	27.8 ± 1.8	30.5 ± 0.7	0 a¶	0 a	0 a	0 a
	16	30.3 ± 0.8	28.5 ± 1.3	29.5 ± 0.9	30.3 ± 0.5	0 a	0 a	0 a	0 a
	32	31.0 ± 2.4	25.5 ± 0.3	27.8 ± 2.0	21.5 ± 1.9	0 a	0 a	0 a	16 b
	64	Not done	Not done	Not done	Not done	Not done	Not done	Not done	Not done
2	0	9.0 ± 6.1	6.5 ± 5.6	12.0 ± 5.5	16.3 ± 5.1	61 dc	72 d	48 bcd	23 abc
	16	18.5 ± 2.5	25.3 ± 1.4	18.0 ± 0.7	21.3 ± 0.9	20 abc	0 a	23 abc	9 ab
	32	22.5 ± 2.1	19.8 ± 1.3	17.5 ± 2.2	20.8 ± 2.7	3 a	15 ab	25 abc	11 ab
	64	22.3 ± 1.9	20.5 ± 2.3	19.9 ± 2.2	22.8 ± 1.8	4 ab	12 ab	17 a	2 a

¶ Only % dandelion control was analyzed using Duncan's multiple range test; different lower case letters for all fractions and rates within a trial represent significant differences at P=0.05

between 65-80% of its macrocidin A content when compared to the reference samples (**Fig. 1**). The TGAI is made by grinding the fermented fungus to coarse flour and has a wide range in particle sizes, while EP is made by grinding the fermented fungus to fine flour followed by processing to a uniform granule. The finely ground EP appeared to release slightly more macrocidin A than the coarsely ground TGAI. However, the response of the EP to leaching was more variable between trials than that of the TGAI, which was consistent.

This study showed that macrocidins are partially soluble in water. This helps to explain the symptoms observed in the field when the bioherbicide is applied to the soil and how macrocidins released into the soil are able to be taken up by the roots of the plants. Microscopic studies (data not published) have also shown that *P. macrostoma* is capable of growing out from the granules applied in the soil, penetrating the roots of plants, and growing parallel to the vascular system, where it may be capable of releasing macrocidins to the water in plant cells.

Soil systems

1. Efficacy of TGAI and leachate with a greenhouse soil mix

Greenhouse soil mix treated with the TGAI of *P. macrostoma* at rates between 16-64 g m^{-2} and leached with 1 L of water prior to being sown with dandelion failed to reduce the number of dandelions per pot with the final numbers of dandelion not significantly different to that of pots devoid of added bioherbicide (**Table 4**). Hence, there was negligible dandelion control after leaching with bioherbicide activity lost from soil. In contrast, soil treated with TGAI at the same rates but not subject to leaching had few if any dandelions present after 21 days resulting in 98-100% dandelion control. These results demonstrated that in a greenhouse soil mix the bioherbicide (at rates of 16 and 64 g m^{-2}) was inactivated by 125 mm of water.

Leachate water fractions collected subsequent to percolation through bioherbicide treated greenhouse soil mix displayed little bioherbicidal activity when applied to untreated pots sown with dandelion (**Table 5**). In Trial 1, there was no dandelion control with fractions F1-F3, while fraction F4 produced in only 16% dandelion control at 32 g m^{-2}. In Trial 2, leachate that did not pass through the bioherbicide had more phytotoxic properties in fractions F1 and F2 than leachate that has passed through the bioherbicide. In this case, phytotoxicity was reduced with increasing fractions of water. Hence, in greenhouse soil, macrocidins were not released in the water, nor did their activity persist in the soil, suggesting that macrocidins were either bound to ingredients in the soil mix or underwent rapid chemical or microbial degradation.

2. Efficacy of TGAI and leachate with three soil types

The soil system studies were repeated to compare the greenhouse soil mix with clay and sandy-loam soils using a higher bioherbicide rate of 128 g m^{-2} and a greater volume of water for leaching. Dandelions planted into source pots that received the equivalent of 250 mm of precipitation had significantly reduced weed control when compared to the unleached source pots (**Fig. 2**). The clay soil and the greenhouse soil mix contained similar levels of bioactivity with approximately 55-60% dandelion control, however the bioactivity in sandy loam resulted in only 10% dandelion control. Source pots that were not treated with the bioherbicide, both with and without leaching, produced a low level of phytotoxicity (about 10-20%). The greenhouse soil mix and the sandy loam soil had the greatest background phytotoxic activity. Since the soil used in the greenhouse mix was identical to that in the same sandy loam soil, additives in the greenhouse soil mix likely increased its phytotoxicity above that of the sandy loan alone

After removing the dandelions from the source pots, a further 125 mm of simulated precipitation was leached through the soil and the source pots re-sown with dandelion. The replanted source pots that had initially been treated with the bioherbicide but had not undergone leaching exhibited 90-100% dandelion control (**Fig. 3**). However, pots containing the bioherbicide that had undergone leaching had significantly reduced dandelion control. In clay soil the % dandelion control was 30%, while control in greenhouse soil mix and sandy loam was 60 and 10%, respectively. The replanted source pots that were not treated with

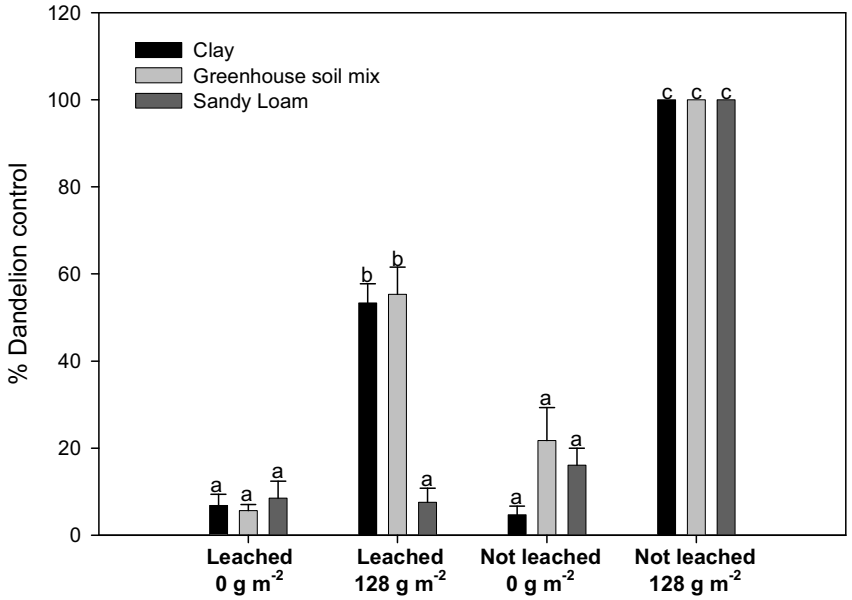

Fig. 2 The effect of leaching the equivalent of 250 mm of precipitation through three soil types containing two rates of the bioherbicide *Phoma macrostoma* on the % dandelion control. The figure shows the combined mean and standard error of 3 trials. Treatments with no error bars had no variation. Lower case letter represent significant differences among treatments using a Duncan's multiple range test at P=0.05.

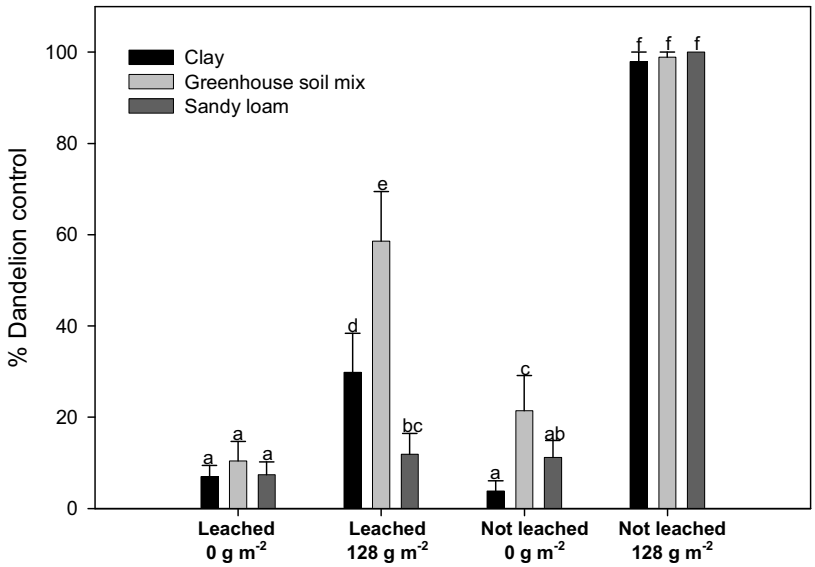

Fig. 3 The effect of leaching the equivalent of 375 mm of precipitation through three soil types containing two rates of the bioherbicide *Phoma macrostoma* on the % dandelion control. The equivalent of 250 mm of precipitation was initially applied and then 4 weeks later another 125 mm was applied. Weed control was estimated at 3 weeks after the second round of leaching. The figure shows the combined mean and standard error of 3 trials. Treatments without error bars had no variation. Different lower case letters among treatments represents significant differences using a Duncan's multiple range test at P=0.05.

the bioherbicide showed variable levels of background phytotoxicity. The untreated pots that were leached reduced dandelion by 5-10%, regardless of soil type. The untreated pots that were not leached also had background phytotoxicty, with clay having the least phytotoxicity followed by sandy loam and greenhouse soil mix.

Leachate fractions F1-F6 from each soil type were tested for their ability to control dandelions by comparing the fractions that had passed through soil containing bioherbicide to those fractions that were passed through soil in the absence of the bioherbicide (**Fig. 4**). When the bioherbicide was present, fractions F1-F3 significantly reduced dandelion in clay soil when compared to untreated clay controls devoid of bioherbicide. The % dandelion control with these fractions was 40% with F1, 80% with F2, and 65% with F3. The level of control then declined to 10% with F4, while fractions F5-F6 provided negligible weed control, which was not significantly different than that of the controls. With the greenhouse soil mix, dandelion control arising from fractions F1-F4 was negligible. Some differences between treated and untreated pots were apparent in fraction F5 with dandelion control equaling 30%. However, there were no differences between the treated and untreated pots with fraction F6. These results supported the observations with fractions F1-F4 from the initial study using the greenhouse soil mix. With the sandy loam, differences between the treated and untreated pots were apparent with fractions F1- F5, with the peak of activity equal to 65% dandelion control observed with fraction F3. With fraction F6, untreated pots once again showed more phytotoxicity than those treated with the bioherbicide.

Some differences were observed among the initial and repeated trials using greenhouse soil mix. The initial trials that had lower rates of bioherbicide application and lower water volumes showed a reduction in bioactivity in the soil and negligible activity in the leachate. The repeated trials that comprised twice the rate of bioherbicide and double the water volume had reduced bioactivity in the soil from un-

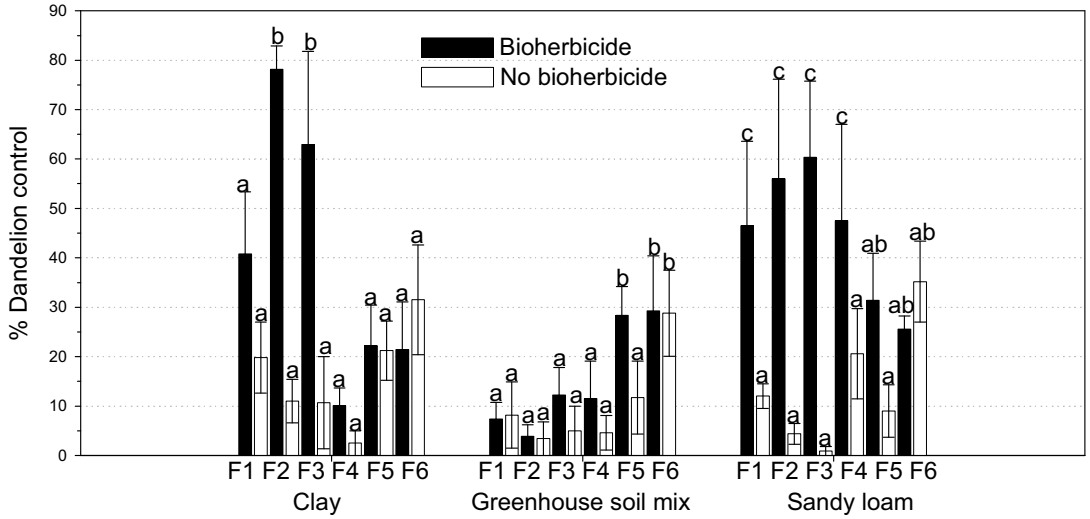

Fig. 4 The effect of leachate fractions F1-F6 passed through three soil types containing either 0 or 128 g m^{-2} of the bioherbicide *Phoma macrostoma* on % dandelion control. The figure represents the combined mean and standard error of three trials. Different lower case letters among fractions within a soil type represent significant differences using a Duncan's multiple range test at P=0.05.

leached pots, but still negligible bioactivity in the leachate. Hence, macrocidins were likely released from the bioherbicide in the soil and utilized by the plants, bound by some ingredient of the soil mix, or rapidly degraded.

These studies showed that larger volumes of water will reduce the efficacy of the bioherbicide, but this effect was dependent on soil type. Macrocidins are released by water in clay and sandy loam soils mostly in the early fractions. To provide perspective, fraction F1 received the equivalent of 63 mm of precipitation. As discussed previously, this was similar to the average monthly rainfall for June or July in Saskatoon. Field trials conducted in Saskatoon in 2002, found no off-site effects due to bioherbicide application (Zhou *et al.* 2004). The monthly precipitation in this area at that time was 54 mm in June, 71 mm in July, and 82 mm in August. The cumulative effects of actual rainfall in that year were similar to the amounts collected in fractions F1-F3 from the current study. Hence, no adverse effects as a result of macrocidins being released in soil water should be expected to occur to nontarget plants under normal levels of precipitation. While we did not recombine the fractions to see if the bioactivity was diluted with increasing water volume, a dilution effect is highly probable since the efficacy of the bioherbicide is dependent on the dose (Zhou *et al.* 2004).

Soil type had an impact on the efficacy of the bioherbicide. The clay and sandy loam soils differed from the greenhouse soil mix by approximately twice the organic content (**Table 1**). The greenhouse soil mix which contained sandy loam soil differed from the sandy loam itself by the presence of a sphagnum peat moss and vermiculite mixture that changed the % organic matter, % carbon, and pH. The organic content of soil may restrict the movement of the bioherbicide by acting as trap or filter-bed for the collection of metabolites and/or fungal biomass, not unlike that reported by Hepple (1960) for different soil horizons. In that report, spores of *Mucor ramannianus* Möller were easily washed through sandy A_1 and A_2 soil horizons but failed to move through the more compacted B_1 horizon, which effectively acted as a filter for the collection of spores. In our experiments, soils containing the sandy loam retained less bioactivity than clay soils, corroborating this concept. Vanninen *et al*. (2000) noted that fungal persistence in soil was not solely dependent on climatic conditions, but soil type in association with specific moisture, temperature and biological characteristics. In our experiments, efficacy was affected by soil type, soil moisture, and the biological/physical properties of the TGAI and EP.

3. Fungal DNA amplification from soil

Soil fractions taken both from leached and unleached pots containing greenhouse soil mix were tested for the presence of fungal DNA using strain specific PCR primers. DNA was only detected in soil from the positive control series at 125 g m^{-2} and the positive fungal DNA control (data not shown). While this meant that the persistence in soil of the solid portion of the formulation could neither be confirmed nor refuted, the dandelion bioassay was more effective at detecting the presence of the bioherbicide than was the soil DNA testing regime at detecting the fungal material.

In field soil, the abundance of fungi applied to the soil surface is thought to be affected by three factors; biodegradation, physical weathering and percolation, the latter being of minor importance (Fargues *et al.* 1983). All of these parameters would also affect a soil-applied bioherbicide. Hepple (1960) noted that a sand column which had been allowed to drain while it contained a spore suspension retained 17 times as many spores as did the same sand column under water-logged conditions. The reason for spore retention despite prolonged washing was attributed to the strong adhesion of spores to sand grains as a result of electrical charge. Although spores may not percolate through soil (and be washed away) water may change the pattern of distribution in the soil. Burges (1950) found that a suspension of wettable spores (i.e., from fungi that produce a mucilaginous coating around the spores) were more readily distributed throughout a column of sand than a suspension of dry spores (i.e., from fungi that produce dry, airborne spores) which stayed concentrated near the top of the column. Burges also found that water volume changed the distribution pattern, with lower water volumes having both horizontal and vertical movement while larger water volumes having primarily vertical movement. Therefore leaching may change the distribution of the TGAI from a mass layer on the soil surface to a more homogenous distribution throughout the soil volume, subsequently lowering DNA concentrations in the soil below the level of detection. The presence of humic acids, which are major constituents of soil organic matter, may also have affected the efficiency by which fungal DNA was isolated and/or amplified from the soil, as humic acids have been shown to interfere with the activity of restriction endonucleases (Tebbe and Vahjen 1993). In hindsight, testing of dandelion roots for the presence of DNA, demonstrating colonization, may have yielded more favorable results.

The plating of leachate fractions on agar was initially attempted with the greenhouse soil mix, but recovery was

less than 2% and there was a high level of contamination with bacteria and other fungi from the soil. This poor level of recovery of was not surprising since Zhou *et al.* (2004) showed that *P. macrostoma* is a slow growing organism in the presence of other fungi and can take up to 21 days before colony identity can be confirmed. The organic matter content of the greenhouse soil mix may also affect the rate of fungal biodegradation in the soil as a function of associated increases in microbiological activity (Fargues *et al.* 1985). In our study high soil moisture may have increased the biological activity of the soils, facilitating biodegradation that could then influence recovery in subsequent leachate fractions. Since the lack of a selective growth medium and high level of contamination interfered with the growth and identification of the fungus, this approach was abandoned. However it did demonstrate that the fungus may be carried through the soil by water, thus supporting the concept that the bioherbicide may have been redistributed throughout the soil volume. Hepple (1960) noted that if water movement does play a role in the vertical movement of fungal propagules, it is likely to be over short distances only, and rarely occur in nature, except under exceptional circumstances.

CONCLUSIONS

Awareness of how a bioherbicide will perform under a range of environmental conditions is important when assessing the risks microbial pest control products pose to people and the environment. Extreme environmental conditions, like heavy rainfall, may facilitate movement of fungal biomass and water soluble metabolites throughout the soil profile, thereby contributing to seepage and off-site contamination. This study evaluated the efficacy of the bioherbicide *P. macrostoma* under simulated rainfall conditions to assess whether bioactivity is retained in the soil at the site of application or whether bioherbicidal metabolites would be soluble in water and therefore amenable to off-site movement via precipitation, thereby posing a risk to non-target, off-site hosts. These studies simulated water volumes equivalent to 25-250 mm of rainfall. The results showed that water (as rainfall) has the capacity to affect efficacy and fate of the bioherbicide in different soils. In all soil types, leaching reduced the ability of the bioherbicide to control dandelion. Clay soil, in comparison to sandy loam and greenhouse soil mix, retained the most bioherbicidal activity. HPLC studies showed that the main bioherbicidal metabolite (macrocidin A) was soluble in water with up to 80% of the macrocidin content in the TGAI being released by the addition of 75 mm of water. Leachate fractions also showed bioactivity. The majority was contained within fractions F1-F3 with much less activity detected in fraction F4 and negligible activity in fractions F5-F6. This pattern of macrocidin release may be beneficial in a situation where the bioherbicide was applied by accident as large volumes of water clearly dilute the bioherbicide, inactivating the macrocidins. However, the impact of water on the distribution of the living component remained in doubt.

This work implies that water facilitates the release of macrocidins from the TGAI and EP formulations allowing these compounds to be taken up by the roots of exposed plants. Repeated rainfall events over the season continue to release macrocidins into the soil with the greatest quantities being released in the first 25-75 mm of rainfall. Macrocidin then continue to be released in conjunction with additional rainfall events, but quantities are negligible such that once precipitation is equivalent to 250 mm, macrocidin release ceases. When soils are not saturated such as at field capacity or drier, rainfall at such volumes is inconsequential as bioactivity remains on-site. However, in saturated soils, macrocidins may be released in the water to be carried off-site via run-off. Hence it is recommended that this bioherbicide should not be applied when the soil is saturated.

A number of factors mitigate the prospect of off-site effects due to run-off water. For example, the infrequency of extreme rainfall events, combined with the intensity and quantity of rainfall prior to the occurrence of an extreme event plays important roles. Similarly, the rate of bioherbicide application alleviates risk to non target plants as lower rates invariably produce leachate with less bioactivity. Also, the risk of off-site effects is not equal at all locations because characteristics of soil type, texture, and organic matter determine whether macrocidins are released, bound, or degraded in soil. The rate of macrocidin release over time and the total water volume is an integrated mitigation factor such that approximately 80% of macrocidins are released into the first 75 mm of water. Additional water over and above this volume dilutes the bioactivity which shows a strong dose response.

Based on the results of this study and the mitigating risk factors, we conclude that the bioherbicide will retain its efficacy in the soil at the point of application and that the passage of macrocidins off-site will be negligible under normal levels of precipitation in unsaturated soils. While the efficacy of the bioherbicide may be reduced in the soil in the aftermath of extreme rainfall events, the aforementioned factors mitigate the impact of macrocidins present in run-off water, thus reducing environmental risk.

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