Bioherbicides for forestry: development of some procedures for bioassay of phytotoxins

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Summary

Laboratory and greenhouse experiments were carried out with extracts of three fungi, *Fusarium oxysporum*, *Cylindrocarpon destructans*, *Collectotrichum dematium* and a commercially manufactured phytotoxin, bialaphos and its analog, glufosinate. These materials were bioassayed on leaf disks of three forest weeds (fireweed, red alder and thimbleberry) and on whole plants of lettuce, soybeans, duckweed and thimbleberry. Results demonstrated that some phytotoxins especially those from *Cylindrocarpon* showed potential for forest weed control. However, the greatest herbicidal activities were obtained from bialaphos and glufosinate. The implications of these findings in relation to biological control of forest weeds by mycoherbicides and natural herbicides (microbial phytotoxins) is discussed.

Introduction

Unwanted vegetation interferes with the productivity of forests and must be controlled intelligently in order to meet the objectives of the plantations (Walstad and Kuch 1987). The competing vegetation reduces yield and increases the cost of production of conifer plantations. Of the various methods of forest weed control, chemical herbicides are rated as the most efficacious and cost-effective tool (Sundaram and Prasad 1984, Row 1987). However, owing to growing environmental concerns and opposition by the public, chemical herbicides are gradually being phased out of forestry and newer ecologically acceptable and environmental benign methods such as biological control, are being tested (Dorworth 1990, Wall 1990, Wall *et al.* 1992).

A promising option is the deployment of bioherbicides whereby deliberate use of natural pathogens to suppress or reduce the population of a weed is advocated (Watson 1989). Some success in control of agricultural weeds by Collego® and DeVine® has been reported (Templeton 1982, Ridings 1986) and the possibility exists that a new mycoherbicide (*Chondrostereum purpureum*) might soon be registered for weed management in forestry (de Jong *et al.* 1990). Most of these microbial herbicides are believed to operate by production of phytotoxins (Hasan and Ayres 1990, Jones *et al.* 1988, Graniti 1972).

There are advantages of using these fungal biorationals rather than the parent (whole) organism in that they are biodegradable and dissipate easily in the environment, i.e., do not persist as spores. For example, there is a possibility that the whole organism of a bioherbicide may serve as a reservoir of infection for other useful and economic crops within 500 metres (de Jong *et al.* 1990). Therefore, it was considered worthwhile to investigate the herbicidal properties of some biorational compounds (phytotoxins) under laboratory and greenhouse conditions. This report describes, (a) the preliminary results obtained from phytotoxins extracted from three pathogenic fungi of native forest weeds together with screening trials of a commercially manufactured natural herbicide, bialaphos and its analog, glufosinate, and (b) some bioassay procedures for determination of phytotoxic properties of these toxins.

Materials and methods

Culture of fungi

Stock cultures of *Colletotrichum dematium*, *Cylindrocarpon destructans* and *Fusarium oxysporum* were isolated from native weeds (*Rubus parviflorus* Nutt. and *Epilobium angustifolium* L.) by Dr. S. Shamoun of this lab and were grown on a standard potato-dextrose-agar (PDA) Czapek media (Difco Manual 1953) and on rice grains according to procedure developed by Abbas *et al.* (1984).

Extraction of toxins

The procedure for preparation of crude fungal-rice extracts and filtrates from the Czapek medium was adopted from Abbas *et al.* (1984). To ensure filtrates were free of mycelia, aliquots were plated on PDA and observed for 96 hrs for growth of no organisms.

Bioassay methods

Determination of phytotoxic properties of the filtrate was carried out by using a series of bioassay procedures:

(a) Leaf disc method

Healthy and vigorous leaves of red alder (*Alnus rubra* Bong), fireweed (*Epilobium angustifolium* L.) and thimble-berry (*Rubus parviflorus* Nutt.) were obtained from uniform plants grown in the greenhouse and discs punched out of their fully developed leaves with a cork borer (size 8) and transferred to water. Discs were surface sterilized by dipping in 95% ethyl alcohol for 30 seconds followed by three rinses with sterile distilled water. Ten discs were aseptically transferred into a sterile glass petri dish and, using a syringe, 10 mL of sterile distilled water and rice filtrate and, fungal extract filtrates were added to these petri dishes through a 0.02 µm millipore filters (Nalgene) to ensure that the supernatant was sterile. Controls consisted of both sterile extracts and distilled water.

All manipulations were carried out in an inoculation hood which was earlier sterilized by ultraviolet light. All discs (treated and untreated) were incubated in a growth chamber set at 25±1°C, 1200 f.c. (16/8 hrs) and 70±15 relative humidity (r.h.). After 5 days, the phytotoxic effects were monitored visually and by a scoring technique (Wall and Shamoun 1990). All experiments were replicated four times and the average observation of a treatment was derived from 40 leaf discs.

(b) Whole plants of lettuce (*Lactuca sativa* L.) and soybeans (*Glycine max* L.) method

Healthy lettuce and soybean seeds were obtained from a local store (A.E. McKenzie Co. Ltd. Victoria) and grown in the greenhouse for 25 days in a soil mixture of peat-vermiculite with low rate of fertilizers NPK (10:5:5). The plants were grown at about 25+1°C, 3000 f.c. (16/8 hr) and 75±15 r.h. When the plants were 25 days old they were sprayed with rice filtrate and culture filtrates of three fungi. Again, controls were both sterile rice filtrates and distilled water. An aerosol sprayer was used to drench the leaves until run-off occurred. All treatments were then returned to the same greenhouse chamber and observed for symptoms for 3–8 weeks. Phytotoxicity was monitored visually and by determination of changes in total dry weights of root and shoot and number of floral and vegetative buds. All experiments were replicated (seven replicates/treatment).

(c) Duck weed (*Lemna minor* L.) method

Various investigators have utilized this free-floating weed for bioassay of herbicides, growth-regulators and toxins (Prasad and Blackman 1965, Prasad 1989, Watson 1989). The duckweed was collected from Beaver-Elk Lake, Victoria, British Columbia and grown in Hoagland solution under controlled conditions in a growth chamber at 23±1°C, 1200 f.c. (16/8 h) according to a procedure outlined by Prasad (1989). When the colony was established, healthy and vigorous growing fronds (10) were treated with full strength (100%) and a diluted series (0, 10, 25 and 50%) of filtrates and allowed to grow for two weeks. There were five replicates of each treatment and solutions were changed periodically to minimize contamination. Phytotoxic effects were monitored by recording discoloration of fronds

as well as by final weighing the colonies.

(d) Seedlings of thimbleberry method

Seedlings of thimbleberry were raised from a seed source obtained from the Ministry of Forests, Prince George, British Columbia. The appropriate seed treatment, chilling and stratification requirements were worked out in our greenhouse and the seedlings were grown in the same way as the lettuce and soybeans plants. Four-month old seedlings with 6–8 leaves were employed for monitoring the effects of three fungal filtrates and the commercial phytotoxin, bialaphos and its analog, glufosinate. Bialaphos (Herbiace®) was obtained from Meiji-Seika-Kasha Ltd. (4-16-Kyobashi-2, Chome-ku Tokyo-104) Japan and contains 20%-amino hydroxy-phosphovinyl-butyryl-alanine as active ingredient (a.i.), and 80% filler. The active ingredient is extracted from *Streptomyces viridochromogenes*. Glufosinate (Ignite®, Hoe 039866) was supplied by Hoescht Canada Ltd. Regina, Sask. and is a structural analog of bialaphos. Its active ingredient is 20% aminohydroxy-phosphovinyl-butyric acid. Apparently the alanine moeity is deficient in glufosinate. Appropriate concentrations of these materials were sprayed on foliage of thimbleberry to the point of run-off and the effects were recorded after three and eight weeks. There were seven replicates/treatment and some experiments were repeated.

(e) Experimental design

All experiments were replicated and randomized. Duncan Multiple Range Test (DMRT) was employed to test the relative effects of treatments as opposed to checks (Snedecor 1957).

Results and discussion

Phytotoxic effects on leaf disc

All three filtrates from the three fungi brought about a similar qualitative effect on leaf discs of alder, fireweed and thimbleberry. Irrespective of test species, the leaf discs became discoloured when exposed to the fungal phytotoxins for five days. Leaf discs in distilled water alone remained greener for longer periods (5–7 days) than those in rice filtrate control which showed some contamination. Generally leaf discs bathed in the fungal extracts started losing green colour 30 hrs after treatment and by the end of 72 hrs they were bleached. Therefore, it seems attractive to speculate that these phytotoxins attacked the chlorophyll of leaves of all three species of weeds and their precise modes-of-action have yet to be elucidated. It is also probable that these phytotoxins like other xenobiotics ruptured cellular membranes, caused leakiness (Prasad 1989) and promoted the loss of metabolites and chlorophyll from the leaf discs.

The fungal extracts from the Czapek media also exhibited a similar response on the leaf discs as measured by the scoring technique (Table 1). In general, all culture filtrates induced the same pattern and degree of phytotoxicity on the three weeds.

The effects of filtrates obtained from rice grains on leaf discs of three weed species are described in Table 2. It is evident that *Cylindrocarpon* extracts show greater phytotoxicity than those of *Fusarium*.

Clearly, the phytotoxins produced on the rice grain appeared to be more effective than those formed in the Czapek medium. Because of this, it was decided to omit the PDA and Czapek media and only rice grains were used for culture of these fungi.

Phytotoxic effects of toxins on soybean plants

Preliminary bioassay tests revealed that soybean seedlings were very sensitive to these fungal extracts. So a detailed analysis of the effects of three fungal extracts on soybean plant growth, (height, shoot, leaves and root) is presented in Table 3.

As can be seen, *Cylindrocarpon* toxins seem to be most effective in reducing the leaf and root growth in soybean seedlings. The toxic effect was so potent that the treated seedlings remained stunted and did not generate any new leaves or buds for up to six weeks. Likewise, the root growth was also greatly retarded. This is of great interest since growth potentials are localized in the buds and roots and any interference with cell-division is likely to produce such drastic effects. This experiment was repeated three times and the same results were obtained each time, confirming the selective mode-of-action of *Cylindrocarpon* toxins on apical meristems. However, when seedlings of thimbleberry were sprayed with this filtrate, the growth was initially arrested, but after one week it resumed and by the end of six weeks there were no significant differences between control and treated plants. Under the conditions of

Table 1. Influence of culture filtrates of three fungi grown of Czapek medium on growth of leaf discs five days after the treatment.

Treatment	Phytotoxicity Rating[1]		
	Alder[2]	Fireweed	Thimbleberry
Control - water	0.0[a]	0.0[a]	0.0[a]
Control - filtrate	0.0[a]	0.0[a]	0.0[a]
Fusarium	1.5[b]	2.0[b]	1.0[b]
Cylindrocarpon	1.5[b]	2.0[b]	1.5[b]
Colletotrichum	1.5[b]	2.0[b]	1.0[b]

[1] 0 – healthy; 5.0 – dead (Wall and Shamoun 1990)

[2] Means in the same column followed by the same letter are not significantly different (P<0.05): Duncan Multiple Range Test.

Table 2. Phytotoxic effects of culture filtrates of three fungi grown on rice grains on leaf disc growth five days after treatment

Treatment	Phytotoxicity Rating[1]		
	Alder[2]	Fireweed	Thimbleberry
Control - water	0.0[a]	0.0[a]	0.0[a]
Control - rice filtrate	0.0[a]	0.0[a]	0.0[a]
Fusarium	1.5[b]	2.0[b]	2.4[b]
Cylindrocarpon	2.4[c]	3.0[c]	3.5[c]
Colletotrichum	2.5[c]	3.0[c]	3.1[c]

[1] 0 – healthy; 5.0 – dead (Wall and Shamoun 1990)

[2] Means in the same column followed by the same letter are not significantly different (P<0.05): Duncan Multiple Range Test.

Table 3. Effects of fungal extracts from three fungi on soybean growth six weeks after treatment.

Treatment	Phytotoxicity				
	Height (mm)	Buds (#)	Leaves (#)	Stem (g)	Root (g)
Control - (rice filtrate)	223.9[a]	24.43[a]	7.71[a]	3.05[a]	0.805[a]
Fusarium	219.9[a]	20.1[a]	6.86[a]	2.38[b]	0.621[b]
Cylindrocarpon	104.7[b]	0.0[b]	0.0[b]	0.77[c]	0.138[c]
Colletotrichum	217.7[a]	24.1[a]	7.71[a]	2.34[b]	0.594[b]

[a] Means in the same column followed by the same letter are not significantly different (P<0.05): Duncan Multiple Range Test.

these experiments, soybeans were more sensitive than thimbleberry plants. Whether there was a differential penetration and translocation of the toxin in these species, remains to be investigated.

Phytotoxic effects on lettuce and duckweed

The results obtained after treatment of lettuce seedlings and the duckweed with extracts of three fungal material were not so dramatic as with the soybeans. Lettuce leaves showed localized burning and stunting of apex initially, but two weeks after treatment plants recovered and resumed growth. Duckweed fronds also showed variable responses (bleaching, buckling, necrosis of fronds) to three filtrates from *Fusarium, Cylindrocarpon* and *Colletotrichum* but it was difficult to maintain their colony growth aseptically for longer periods. Organic nutrients from rice filtrate induced prolific growth of algal and bacterial population and masked the expression of real phytotoxic effects. Therefore, the filtrates must be purified in order to use *Lemna* as a bioassay test.

Phytotoxic effects of bialaphos and glufosinate

These two commercial products when sprayed on the thimbleberry seedlings at 0, 10, 40, 100, 1000 ppm, produced visible effects after one week. By three weeks, plants treated with 1000 ppm were completely browned and dead; no resprouting had occurred by eight weeks. Concentrations lower than 1000 ppm were not as effective, as recovery and resprouting from the adventitious buds took place. Sufficient translocation of the materials is required for killing sub-terranean parts (rhizomes) and judging from the extent of resprouting, it appeared that lower dosages, did not translocate as rapidly as the highest concentrations. That bialaphos translocated in red raspberry to provide a high degree of residual toxicity was recently reported by Jobidon (1990). Duke and Lydon (1987) have also discussed the mechanism of action of bialaphos in detail. Further tests are in progress to evaluate the potential of these two products on efficacy of other weeds and crop tolerance of conifer species.

In conclusion, fungal extracts of three fungi (*Fusarium oxysporum, Collectotrichum dematium* and *Cylindrocarpon destructans*) exhibit phytotoxin properties on leaf disc of alder, fireweed and thimbleberry and whole plants of lettuce and soybeans. *Cylindrocarpon* extracts were highly phytotoxic to soybean seedlings, but showed only weak bioherbicidal impact on the major target weed (thimbleberry). This would probably limit use of this mycoherbicide on *Rubus* species. Two commercially produced phytotoxins (bialaphos and glufosinate) offer great promise for control of thimbleberry but further screening on crop tolerance of western conifer species is required before any substantive claim can be made. For the bioassay of *Cylindrocarpon* phytotoxins, soybean and for the bialaphos, thimbleberry seedlings provide sensitive screens while leaf discs from alder, fireweed and cultures of duckweed exhibit microbial contaminations and may not be suitable for tests until fungal filtrates are purified.

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