Bull. Environ. Contam. Toxicol. (1989) 42:835–839

Environmental Contamination and Toxicology

Effects of the Herbicides 2,4-D, Glyphosate, Hexazinone, and Triclopyr on the Growth of Three Species of Ectomycorrhizal Fungi

D. Estok,[1] B. Freedman,[1,2,3] and D. Boyle[2]

[1]Department of Biology and School for Resource and Environmental Studies, Dalhousie University, Halifax, Nova Scotia, Canada, B3H 4J1 and [2]Nova Scotia Research Foundation Corp., 100 Fenwick St., Box 790, Dartmouth, Nova Scotia, Canada, B2Y 3Z7

The benefits imparted to host plants by mycorrhizal fungi are well documented (Harley and Smith 1983). The most frequently cited benefits are increased nutrient uptake and improved resistance to stress. These symbiotic associations are necessary for the proper growth and development of most vascular plants, including commercially important gymnosperm trees.

In recent years, herbicides have come into increasing use as a silvicultural tool. However, little is known about their effects on important non-target organisms such as mycorrhizal fungi. This information is required in order to ensure that forest ecosystems are not damaged by herbicide use. Here we report the results of studies of the toxicity of four herbicides to representative species of ectomycorrhizal fungi that infect forest trees. The herbicides 2,4-D and glyphosate are presently used in silviculture in Canada, while triclopyr and hexazinone are unregistered for forestry purposes. All four of the herbicides are registered for forestry purposes in the United States.

MATERIALS AND METHODS

The three species of ectomycorrhizal fungi used were: Cenococcum geophilum Fr., Pisolithus tinctorius (Pers.) Coker and Couch, and Hebeloma longicaudum (Pers.: Fr.) Kumm. Stock cultures of these were maintained at 25° C in the dark on Modified Melin-Norkrans (MMN) agar medium (Marx 1969).

Four herbicides were tested, all formulated as emulsifiable concentrates: i) Garlon 4 - active ingredient (a.i.) triclopyr [(3,5,6-trichloro-2-pyridinyl) oxyacetic acid], at 480 g/l as a butoxy ethyl ester in a 38.4% petroleum distillate (including kerosene) solvent; ii) Roundup - a.i. glyphosate (N- phosphonomethyl-glycine), at 336 g/l in aqueous solution; iii) Chipman Dandilion Killer - a.i. 2,4-D (2,4-dichlorophenoxyacetic acid), hexazinone [3-cyclohexyl-6-(dimethyl-amino)-1-methyl-1,3,5-triazine-2,4(1H,3H)-dione], at 250 g/l as a miscible liquid in ethanol solution.

A stock solution of each herbicide was filter-sterilized using cellulose acetate (triclopyr) or nitrocellulose (2,4-D, glyphosate, and hexazinone) membrane filters (0.45 um). The sterile filtrate of each herbicide was diluted with sterile distilled water to produce a stock solution of 5% a.i. All herbicide formulations were soluble in water except triclopyr, which formed a fine emulsion.

3) Send reprint requests to B. Freedman at the above address.

A MMN agar medium with glucose (pH 5.5) was prepared and autoclaved, and each herbicide solution was added to the molten MMN agar to produce concentrations of 1, 10, 100, 1000, 5000, and 10,000 ppm a.i. The herbicide-agar mixtures were thoroughly mixed and poured into petri plates (25 mL/dish). These were inoculated with a 4 mm-diameter agar disc cut with a flame-sterilized cork borer from the periphery of an actively growing stock culture. Discs were centrally placed, mycelium-side up on the plates. There were four replicates per herbicide-fungus treatment, plus four controls without herbicide. Each plate was wrapped in parafilm, and incubated in the dark at 24° C for 26 days (P. tinctorius) or 48 days (C. geophilum and H. longicaudum).

At the termination of the experiment, the outline of each fungal colony was traced onto a clear acetate sheet. The tracings were cut out, weighed, and the colony area calculated from the known ratio of acetate area : weight (minus the initial area of the inoculation disc).

Each data set (fungus x herbicide) was subjected to an Analysis of Variance. Fisher's Least Significant Difference was used to determine which treatments were significantly different from controls.

RESULTS AND DISCUSSION

Each herbicide significantly reduced the radial growth of each species of ectomycorrhizal fungus at concentrations $\geq$ 1000 ppm (Table 1). Growth was completely inhibited at concentrations $\geq$ 5000 ppm. Cenococcum geophilum was the slowest growing fungus, and its radial growth was least sensitive to the herbicides. Its growth was greater than or did not differ from controls at concentrations $\leq$ 100 ppm of all herbicides, except glyphosate at 100 ppm (9% of control). The other two fungi had considerably higher radial growth rates and were more susceptible to toxic effects at particular herbicide concentrations (except for glyphosate). Neither species showed enhanced growth in the presence of herbicide.

Other studies of the effects of herbicides on ectomycorrhizal fungi and ectomycorrhizae have variously reported toxic effects, no effect, and stimulation, depending on the species, the herbicide, and the dose (reviewed by Trappe et al. 1984). For example, Ibola (1978) found that 2,4-D had little effect on colony growth of three ectomycorrhizal fungi (Tricholoma saponaceum, T. pessundatum, and Amanita citrina) at a concentration of 10 ppm, but it was inhibitory at higher concentrations, and growth was completely suppressed at $\geq$ 1000 ppm. Lake et al. (1981) reported a growth decrease of Scleroderma aurantium in the presence of $\geq$1.0 ppm glyphosate in a liquid medium, while P. tinctorius was either not affected or it was stimulated. Triclopyr and hexazinone had either no effect or they stimulated the growth of these fungi at 0.5 to 10 ppm. Kelley and South (1980) found that the growth of colonies of P. tinctorius was significantly reduced at 1 ppm hexazinone in agar. Chakravarty and Sidhu (1987) found that the growth of cultures of five species of mycorrhizal fungi was significantly reduced by exposures >10 ppm of glyphosate, hexazinone, and trichlopyr.

In typical silvicultural usage, herbicide application rates range from about 1.7 to 5.0 kg/ha for glyphosate (Ghassemi et al. 1981), 0.28 to 10 kg/ha for triclopyr, 0.56 to 9.5 kg/ha for 2,4-D, and 0.56 to 3.4 kg/ha for hexazinone (Sassman et al. 1984). Following such application rates, typical initial residues in the forest floor are 5 to 10 ppm of glyphosate (Rueppel et al. 1977), 4 to 18 ppm of triclopyr (McKeller et al. 1982), 5 to 20 ppm of 2,4-D (Norris 1981), and 4 to 10 ppm of hexazinone (Rhodes et al. 1977). Somewhat larger residues are sometimes observed, but according to Norris (1981) the expected initial residues of 2,4-D in the forest floor only rarely exceed 100 ppm. Therefore, if the application rate

Table 1. Effects of herbicides on mycelial growth of three species of ectomycorrhizal fungi. Data are average colony area (cm2; n = 4; S.D. in parentheses).

ppm	triclopyr	glyphosate	hexazinone	2,4-D
a) Cenococcum geophilum				
0	3.1 (1.3)	3.1 (1.3)	3.1 (1.3)	3.1 (1.3)
1	6.3 (1.9)**	3.8 (1.4)	4.4 (1.3)*	3.3 (1.0)
10	7.1 (0.2)**	2.7 (0.4)	6.7 (0.8)**	3.9 (1.2)
100	3.5 (0.9)	0.3 (0.1)**	3.5 (0.7)	3.4 (0.6)
1000	0.0 (0.0)**	0.1 (0.04)**	0.9 (0.05)**	0.0 (0.0)**
5000	0.0 (0.0)**	0.0 (0.0)**	0.0 (0.0)**	0.0 (0.0)**
10000	0.0 (0.0)**	0.0 (0.0)**	0.0 (0.0)**	0.0 (0.0)**
b) Hebeloma longicaudum				
0	20.5 (6.1)	20.5 (6.1)	20.5 (6.1)	20.5 (6.1)
1	16.4 (1.8)*	19.8 (5.8)	18.2 (1.8)	20.3 (8.9)
10	16.8 (0.9)*	16.1 (2.1)	21.9 (3.4)	15.6 (1.0)
100	7.9 (0.4)**	8.0 (1.0)**	15.7 (1.1)*	6.6 (1.5)**
1000	0.1 (0.02)**	2.3 (0.6)**	3.7 (1.0)**	0.0 (0.0)**
5000	0.0 (0.0)**	0.0 (0.0)**	0.0 (0.0)**	0.0 (0.0)**
10000	0.0 (0.0)**	0.0 (0.0)**	0.0 (0.0)**	0.0 (0.0)**
c) Pisolithus tinctorius				
0	56.6 (0.0)	56.6 (0.0)	56.6 (0.0)	56.6 (0.0)
1	19.5 (1.2)**	30.0 (2.6)**	44.4 (1.5)**	47.6 (2.1)**
10	15.8 (0.3)**	17.4 (3.0)**	41.4 (4.1)**	31.9 (1.6)**
100	5.2 (0.6)**	4.7 (1.3)**	39.5 (1.2)**	0.0 (0.0)**
1000	0.02 (0.02)**	0.0 (0.0)**	6.7 (0.1)**	0.0 (0.0)**
5000	0.0 (0.0)**	0.0 (0.0)**	0.0 (0.0)**	0.0 (0.0)**
10000	0.0 (0.0)**	0.0 (0.0)**	0.0 (0.0)**	0.0 (0.0)**

* significantly different from control at $p < 0.05$
** significantly different from control at $p < 0.01$

falls within the above ranges, it can reasonably be assumed that the expected initial bulk residues of the four herbicides examined here would be less than about 100 ppm in the forest floor.

In our bioassays, we found evidence of inhibition of fungal growth at herbicide concentrations $\leq$ 100 ppm. However, it is important to note that agar medium presents a very different bioassay condition from that experienced at a similar bulk concentration of herbicide in the much more complex and variable environment of the forest floor or soil (Bollen 1961; Kreutzer 1963). In general however, the conditions in agar tend to predispose the bioassay fungi to herbicide toxicity. The most important considerations are:

i) the nutrient-rich, competition-free agar medium presents an ameliorated environment that is specifically designed to enhance fungal growth. The resulting rapid rates of growth and metabolic activity increase the overall uptake of herbicide from the medium, resulting in larger concentrations in hyphae and enhanced toxicity (Marx and Rowan 1981);

ii) laboratory stocks of microorganisms are grown for many generations on enriched media. Because of this "extensive laboratory acclimation" they are relatively susceptible to sudden and adverse environmental change, such as the presence of a xenobiotic chemical in their growth medium (Pritchard and Bourquin 1984);

iii) because agar is essentially an aqueous medium with relatively few barriers to the diffusion of chemicals, the herbicides are characterized by greater availability than in the forest floor or soil, where ion exchange processes immobilize a large fraction of the residues (Norris 1981); and

iv) herbicides in agar are not rapidly degraded by a diverse heterotrophic microflora or by inorganic or photochemical processes, nor are they otherwise dissipated by mass transport mechanisms such as volatilization, leaching, and transport of adsorbed herbicide with eroding particles (Martin 1963; Norris 1981; Sassman et al 1984). This contrasts with the forest floor or soil environment, where the herbicides tested here have a fairly short persistence.

Estimates of the half-lives in soil and the forest floor range from about 2 to 6 weeks for 2,4-D (Foster and McKenchen 1973; Plumb et al. 1977; Fletcher and Freedman 1986) and 3 to 134 days for glyphosate (Ghassemi et al. 1981; Sassman et al. 1984; Morash et al. 1987). There is less information about the persistence of hexazinone and triclopyr, but it appears that the expected half-lives would be less than 2 months (Rhodes 1980; Ghassemi et al. 1981; Sassman et al. 1984).

Acknowledgments. Funding for this research was through an NSERC operating grant to B.F. We thank R.P. McBride and J. Novitsky of the Department of Biology at Dalhousie University for their critical reading of a draft of the manuscript.

REFERENCES

Bollen WB (1961) Interactions between pesticides and microorganisms. Ann Rev Microbiol 15:69-92.

Chakravarty P, SS Sidhu (1987) Effect of glyphosate, hexazinone, and triclopyr on in vitro growth of five species of ectomycorrhizal fungi. J For Path 17:204-210.

Fletcher K, B Freedman (1986) Effects of the herbicides glyphosate, 2,4,5-T, and 2,4-D on forest litter decomposition. Can J For Res 16:6-9.

Foster RH, RB McKenchen (1973) Laboratory incubation studies of chlorophenoxyacetic acids in chernozoic soils. Soil Biol Biochem 5:333-350.

Ghassemi M, L Fargo, P Painter, S Quinlivan, R Scofield, A Takata (1981) Environmental Fates and Impacts of Major Forest Use Pesticides. cited by Sassman et al (1984).

Harley JL, SE Smith (1983) Mycorrhizal Symbiosis. Academic Press, Inc. New York, N.Y.

Ibola C (1978) The influence of 2,4-D on ectotromycorrhizal symbiosis in pine and spruce seedlings. Eur J For Pathol 8:379-383.

Kelley WD, DB South (1980) Effects of herbicides on in vitro growth of mycorrhizae of pine (Pinus spp.). Weed Sci 28:599-602.

Kreutzer WA (1963) Selective toxicity of chemicals to soil microorganisms. Ann Rev Phytopathol 1:101-126.
Lake DB, DJ Ippoliti, CC Brandow, WJ Otrosina (1981) Effect of herbicides on the growth of Pisolithus tinctorius and Scleroderma aurantium in pure culture. p. 62 in: Fifth North American Conference on Mycorrhizae. Aug. 16-21, 1981. Quebec, P.Q.
Martin JP (1963) Influence of pesticide residues on soil microbiological and chemical properties. Residue Rev 4:96-129.
Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. 1. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59:153-163.
Marx DH, SJ Rowan (1981) Fungicides influence growth and development of sensitive specific ectomycorrhizae on loblolly pine seedlings. Forest Sci 27:167-176.
McKellar RL, OE Schubert, BC Byrd, LP Stevens, EJ Morton (1982) Aerial application of Garlon 3A herbicide to a West Virginia watershed. Down to Earth 38:15-19.
Morash R, B Freedman, R McCurdy (1987) Persistence of glyphosate in foliage and litter. in: Environmental Impacts of Silvicultural Glyphosate Use. Environment Canada, Environmental Protection Service. Dartmouth, N.S.
Norris LA (1981) The movement, persistence, and fate of the phenoxy herbicides and TCDD in the forest. Residue Rev 80: 65-135.
Plumb TR, LA Norris, ML Montgomery (1977) Persistence of 2,4-D and 2,4,5-T in chapparal soil and vegetation. Bull Environ Contam Toxicol 17:1-8.
Pritchard FH, AW Bourquin (1984) The use of microcosms for evaluation of interactions between pollutants and microorganisms. Adv Microb Ecol 7:133-215.
Rhodes RC (1980) Soil studies with carbon-14 labelled hexazinone. J Agric Food Chem 28:311-315.
Rhodes RC, RL Krause, MH Williams (1977) Microbial activity in soils treated with hexazinone. Soil Sci 129:311-314.
Rueppel ML, BB Brightwell,JS Chaeter, JT Marvel (1977) Metabolism and degradation of glyphosate in soil and water. J Agric Food Chem 25:517-522.
Sassman J, R Pienta, M Jones, J Cioffi (1984) Pesticide Background Statements. Volume 1.: Herbicides. U.S.D.A. Forest Service, Agriculture Handbook No. 633. Washington, D.C.
Trappe JM, R Molina, M Castellano (1984) Reactions of mycorrhizal fungi and mycorrhiza formation to pesticides. Ann Rev Phytopathol 22:331-359.

Received April 10, 1988; accepted November 5, 1988.