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EFFECT OF SOME SYSTEMIC AND PROTECTANT FUNGICIDES ON GROWTH OF *VERTICILLIUM ALBO-ATRUM*, *RHIZOBIUM MELILOTI* AND LUCERNE SEEDLINGS IN THE LABORATORY

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ABSTRACT

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The effect of six systemic and six non-systemic fungicides on the growth of seven *Verticillium albo-atrum* isolates on PDA was examined at 1 and 10 μg a.i./ml. With the exception of phosethyl aluminium, the systemics inhibited growth, the inhibition being complete at 10 μg benomyl or ectaconazole. At the concentrations tested, protectant fungicides had a very limited efficacy on all isolates. The tolerance of six strains of *Rhizobium meliloti* to the same set of fungicides was also examined; all were highly sensitive to thiram, metiram and maneb, and tolerant to high concentrations of anilazine, chlorothalonil and triadimenol. Fungitoxic properties of benomyl, ectaconazole and triadimenol were mostly not modified after a 6-day exposure of these three systemics to *R. meliloti* in agar. The same three fungicides, applied at 5 μg per seed in a nutrient solution, stimulated nitrogenase activity in lucerne seedlings. At the dosage tested, growth of seedlings was enhanced by benomyl and triadimenol but decreased by ectaconazole.

INTRODUCTION

Verticillium wilt, caused by *Verticillium albo-atrum* Reinke & Berth., is probably the most damaging disease of lucerne (*Medicago sativa* L.) in Europe, and is now occurring in a growing number of areas in North America (Gagné and Richard, 1982). To prevent spreading this disease with seed, and until adapted resistant cultivars are available (Busch and Smith, 1981), all seed lots of lucerne sold in Canada have to be treated with the protectant fungicide thiram at the dose of 250 g/100 kg seed

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(Agric. Canada, 1979). At such a dose, thiram was observed to cause a temporary bacteriostatic effect on *Rhizobium meliloti* (Sirois et al., 1981), the symbiotic partner of lucerne. At doses in the same range, Tu (1981a) also observed an inhibitory effect of thiram on lucerne growth, nodulation and symbiotic nitrogen fixation.

Thiram can successfully control *V. albo-atrum* on seed surface and on trash carried with seed (Isaac and Lloyd, 1957). However, recent reports on the presence of *V. albo-atrum* within lucerne seed (Sheppard and Needham, 1980; Christen, 1982) suggest that the use of a systemic fungicide for seed treatment may be more efficient and suitable in controlling dissemination of the pathogen.

The present work has compared the inhibitory effect of low concentrations of systemic and non-systemic fungicides on the growth of different isolates of *V. albo-atrum* and assessed the level of tolerance of *R. meliloti* strains to the same fungicides. As benomyl, ectaconazole and triadimenol inhibited growth of the fungal isolates, their effect on growth of lucerne seedlings and nitrogenase activity was also studied under laboratory conditions.

MATERIALS AND METHODS

The systemic fungicides used were: benomyl (Benlate, 50% W.P., DuPont of Canada Ltd.), ectaconazole (CGA 64251, 2.5% *w/v* S.C.O., Ciba Geigy Canada Ltd.), furmecyclox (BAS 38908F, 40% W.P., BASF Canada Inc.), phosethyl aluminium (Aliette, 80% W.P., May and Baker Canada Ltd.), RH 5781F (18% *w/v* E.C., Rohm and Haas Canada Ltd.) and triadimenol (TF 3481, 10% D., Chipman Inc.). Non-systemic fungicides used were: anilazine (Dyrene, 50% W.P., Chemagro Ltd.), chlorothalonil (Bravo 500, 50% *w/v* F., Diamond Shamrock Canada Ltd.), iprodione (Rovral, 50% W.P., May and Baker Canada Ltd.), maneb (Agrox NM, 50% D., Chipman Inc.), metiram (Polyram, 7% D., BASF Canada Inc.) and thiram (50% D., Ciba Geigy Canada Ltd.).

V. albo-atrum isolates were from the culture collection of Agriculture Canada research station at Sainte-Foy, and had been originally isolated from lucerne or clovers by C. Aubé, Sainte-Foy (Nos. 171, 191 and 202), J. Gondran, France (Nos. 227, 228 and 229) and J.W. Sheppard, Ottawa (No. 282). The strains A_2, A_3, S_5, S_{14}, V_7 and 3Doa8 of *R. meliloti* were used. The symbiotic effectiveness of these strains was previously described (Bordeleau et al., 1977). The effect of the fungicides on the radial growth of *V. albo-atrum* was evaluated as follows: each fungicide was tested at concentrations of 1 and 10 μg active ingredient (a.i.) per ml of culture medium. Stock solutions of 1000 and 10 000 μg a.i./ml were prepared by suspending the appropriate amount of fungicides in 70% ethanol, and 1 ml of each solution was added to 1 l potato dextrose agar (PDA, Difco) cooled to 45°C. The agar and fungicide were mixed together and 20 ml was poured into each of five petri plates for each fungus isolate. The check

plates received PDA containing 1 ml ethanol per l. The plates were allowed to cool before inoculating their centre with a 7-mm plug taken from the margin of actively growing colonies. The plates were incubated in the dark at room temperature. Colony diameters were measured after 12 days (14 days for culture No. 191).

The level of tolerance of *R. meliloti* strains to fungicides was evaluated using an antibiotic paper disc method. Antibiotic paper discs (12.7-mm diameter, Schleicher & Schuell Inc., Keene, NH) were imbued with 0.1 ml ethanol 70% containing up to 3000 μg of the fungicides and were dried at 30° C. Four discs of different treatments per plate were placed on yeast mannitol agar (Vincent, 1970) surface inoculated with *R. meliloti* strains as outlined by Antoun et al. (1982). Inhibition zones around three replicate discs per treatment were recorded after 2—3 days of incubation at 30° C.

Benomyl, ectaconazole and triadimenol were highly effective in inhibiting growth of *V. albo-atrum* isolates and were retained for further studies. The antifungal efficacy of these three systemics was tested in presence of *R. meliloti* strains by a double layer agar technique (Antoun et al., 1978). The bottom layer was made of 15 ml yeast mannitol agar supplemented with fungicide (10 μg a.i./ml) prepared in ethanol as described earlier, and inoculated at 45° C with 1 ml yeast mannitol broth containing approximately 10^8 *R. meliloti* cells. After a 6-day incubation at 30° C, this first layer was covered with a thin second layer made of 5 ml PDA. The plates were kept 18 h at room temperature to allow diffusion of the fungicides into the top layer which was then inoculated with isolate No. 202 of *V. albo-atrum* used as a detector of fungitoxic effect. Radial growth of the fungus was measured as described earlier.

The effects of the same three fungicides on growth of lucerne seedlings and nitrogen fixation were studied using seeds of lucerne cv. Titan surface-sterilized and grown in plastic pouches as described by Bordeleau et al. (1981). Each growth pouch received 30 ml of sterile Hoagland's nitrogen-free nutrient solution containing 50 μg a.i. of each fungicide (this concentration was calculated on the basis of 5 μg a.i. per seed). One week after sowing, each growth pouch containing ten seedlings was inoculated with 1 ml of a suspension of the strain A_2 of *R. meliloti* containing approximatively 10^9 cells. Each treatment included ten replicates (pouches). The plants were grown for 4 weeks in a controlled environment room under a 16 h light regime of 1.9 klx at 18–20° C and 8 h dark at 11—12° C. Nutrient solution volume was maintained in each pouch by periodical additions of sterile distilled water. At harvest, the shoots and roots of seedlings in each pouch were separated. The shoots were oven dried at 80° C for dry weight determinations and the roots were incubated for 30 min in 60 ml serum bottles containing 0.1 atm acetylene. Ethylene produced, as an indicator of nitrogenase activity, was measured in the bottles with a Tracor 220 gas chromatograph equipped with a hydrogen flame ionization detector kept at 125° C and a column (183 cm long and 3 mm internal diameter) packed with Porapak N (100—120 mesh) operated at 55° C.

TABLE I

Effect of systemic and non-systemic fungicides on the radial growth of seven isolates of *Verticillium albo-atrum* on PDA

Fungicide		Radial growth as a percentage of checks						
Active ingredient	Concentration (μg a.i./ml)	Isolates						
		No. 171	No. 191	No. 202	No. 227	No. 228	No. 229	No. 282
Checks		100 (*49.0*)[a]	100 (*29.7*)	100 (*45.2*)	100 (*40.1*)	100 (*46.1*)	100 (*43.0*)	100 (*45.7*)
Systemic								
benomyl	1	0	47.0	0	0	0	0	5.5
	10	0	0	0	0	0	0	0.2
ectaconazole	1	8.3	15.3	7.5	8.2	8.0	7.8	6.6
	10	0	0	0	0	0	0	0
furmecyclox	1	93.4	103.9	99.5	98.9	94.3	92.9	99.5
	10	16.1	74.6	20.4	25.3	29.4	17.4	40.6
phosethyl aluminium	1	99.5	106.2	106.6	101.1	92.3	91.9	101.0
	10	92.2	103.3	97.9	94.5	88.5	89.3	97.5
RH 5781F	1	68.4	86.7	71.3	61.2	69.9	71.8	72.1
	10	14.1	44.6	13.0	12.0	15.9	15.1	8.0
Triadimenol	1	40.5	87.2	40.6	40.5	38.8	36.2	29.3
	10	4.8	45.8	2.8	5.7	2.3	2.2	1.0
Non-systemic								
anilazine	1	99.1	104.7	102.6	101.1	88.6	82.5	99.5
	10	95.1	102.5	104.2	106.1	88.6	79.7	97.2
chlorothalonil	1	86.2	86.2	83.7	82.0	83.7	76.5	81.1
	10	75.6	74.8	75.4	72.6	62.5	57.7	74.4
iprodione	1	94.6	103.0	96.5	98.0	79.6	77.3	99.1
	10	24.3	64.8	33.9	37.0	66.5	47.5	37.1
maneb	1	101.3	105.2	105.4	105.2	97.0	92.1	100
	10	96.6	98.8	102.8	108.5	91.4	83.1	94.4
metiram	1	91.8	100.7	96.2	94.1	84.4	85.5	97.2
	10	95.8	96.5	100.6	102.4	90.9	82.0	96.2
thiram	1	100.1	109.3	106.3	104.9	91.8	90.1	99.2
	10	95.2	96.0	99.9	102.5	87.8	83.1	98.8
Standard deviation		2.4	3.9	3.1	3.2	3.4	2.8	3.1

[a] Values in italics are the actual radial growth (mm) of check cultures.

RESULTS AND DISCUSSION

Table I shows the effect of fungicides tested on the radial growth of *V. albo-atrum* isolates. None of the protectant fungicides completely inhibited the radial growth of the isolates at the concentrations used. Iprodione at 10 μg/ml was the only non-systemic inhibiting the radial growth of at least one isolate by 50% or more. The limited efficacy of non-systemic fungicides can be ascribed to poor diffusion in agar or more likely to the low concentrations used.

The systemic fungicide phosethyl aluminium was similar to most non-systemic fungicides and did not affect growth of *V. albo-atrum* (Table I). However, the other systemics were effective in inhibiting fungal growth. Benomyl and ectaconazole at 10 μg/ml completely inhibited the radial growth of all isolates. Triadimenol also showed a high inhibitory effect, but was less effective than benomyl and ectaconazole at 1 μg/ml. Furmecyclox and RH 5781F were only effective at 10 μg/ml. The slow growing isolate No. 191 of *V. albo-atrum* was the most resistant to systemic fungicides.

TABLE II

Sensitivity of six *Rhizobium meliloti* strains to systemic and non-systemic fungicides on agar medium

Fungicide	Highest concentration showing no growth inhibition (μg per paper disc)[a]					
	Strain					
	A_2	A_3	S_5	S_{14}	V_7	3Doa8
Systemic						
benomyl	750	1000	1000	3000	250	3000
ectaconazole	500	250	500	500	250	500
furmecyclox	1500	750	750	1500	750	3000
phosethyl aluminium	100	250	100	100	100	250
RH 5781F	1000	500	750	750	1000	3000
triadimenol	3000	1000	3000	3000	1000	3000
Non-systemic						
anilazine	3000	750	3000	3000	1500	3000
chlorothalonil	3000	750	3000	1500	1500	3000
iprodione	1000	1500	1000	1000	750	3000
maneb	50	50	100	25	50	100
metiram	25	25	25	10	25	50
thiram	25	10	25	10	10	25

[a]The maximum tested concentration was 3000 μg per disc.

The six *R. meliloti* strains used were more sensitive to the dithiocarbamate fungicides thiram, metiram and maneb, and showed tolerance to high concentrations of anilazine, chlorothalonil and triadimenol (Table II). Other fungicides had an intermediate level of toxicity to *Rhizobium*. The toxicity of thiram recorded here at concentrations > 25 μg per disc contrasts with the absence of inhibition reported by Tu (1981a) with concentrations up to 500 μg per disc on the one strain tested. The explanation for such a discrepancy might be the range of sensitivity among *R. meliloti* strains. Indeed each of the six strains that we used showed a unique pattern of tolerance to the set of fungicides tested and this property may be of value in strain identification as is the case with antibiotics (Antoun et al., 1982).

Because of their inhibitory effect on growth of *V. albo-atrum*, benomyl, ectaconazole and triadimenol were further investigated. The presence of *R. meliloti* strains did not affect the fungitoxic effect of benomyl and ectaconazole (Table III). This indicates that these two chemicals are not modified by *R. meliloti* or that the products of a possible degradation are also effective. The inhibitory effect of triadimenol on *Verticillium* was slightly decreased by strain 3Doa8 and enhanced by strain A_2 (Table III). In the absence of fungicide, strains A_2, A_3 and S_{14} of *R. meliloti* had a direct inhibitory effect on the fungus. Such an inhibitory effect may be the result of competition for nutrients (Antoun et al., 1978).

After 4 weeks in presence of benomyl or triadimenol applied at 5 μg per seed, the growth of lucerne seedlings was significantly enhanced as indicated by shoots dry weight (Table IV).

Ectaconazole, however, significantly decreased seedlings growth. The three fungicides stimulated nitrogenase activity with triadimenol being

TABLE III

Growth of *Verticillium albo-atrum* (isolate No. 202) on PDA as influenced by benomyl, ectaconazole and triadimenol previously exposed to six *Rhizobium meliloti* strains, as determined by a double layer agar method

Fungicide in the bottom layer[a]	Radial growth of *V. albo-atrum* on the top layer (mm)						
	No *Rhizobium*	Strain					
		A_2	A_3	S_5	S_{14}	V_7	3Doa8
None (check)	46.2a[b]	38.8b	38.8b	40.7a	36.6c	39.7ab	40.2ab
benomyl	0a	0a	0a	0a	0a	0a	0a
ectaconazole	0a	0a	0a	0a	0a	0a	0a
triadimenol	1.4b	0.1c	2.3ab	2.0ab	1.7b	2.1ab	2.8a

[a] 10 μg/ml.
[b] Within each line means followed by the same letter are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

TABLE IV

Effect of benomyl, ectaconazole and triadimenol on growth of seedlings of lucerne and on symbiotic nitrogen fixation measured after 4 weeks

Fungicide[a]	Shoots dry weight[b] (mg per plant)	Nitrogenase activity (nmol ethylene per plant h^{-1})
benomyl	8.26a[c]	30.39b
ectaconazole	4.93c	27.86b
triadimenol	7.71a	38.7a
None (check)	6.37b	12.41c

[a] 5 μg per plant in the nutrient solution.
[b] Average of 100 seedlings.
[c] Within each column means followed by the same letter are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

more significant than the other two. Such a stimulation was reported in soya-bean (*Glycine max* (L.) Merr.) treated with the insecticide diazinon (Tu, 1981b), and in lucerne treated with benomyl at 200 and 500 μg/g seed (Tu, 1981a). Further studies are required to elucidate the nature of the stimulatory effects of pesticides on nitrogenase activity in legumes.

CONCLUSION

Under laboratory conditions, *V. albo-atrum* isolates were more sensitive to low concentrations of systemic fungicides than to protectant fungicides. Among all fungicides tested, the systemics benomyl, ectaconazole and triadimenol were the most fungitoxic, and *R. meliloti* strains were resistant to medium to high concentrations of these three. Fungitoxic properties of these three were mostly not modified by the action of *R. meliloti*, and the fungicides had with one exception beneficial effects on lucerne and nitrogen fixation. Benomyl for one was reported to control seed-borne fungi effectively in soya-bean (Jeffers et al., 1982) and had no inhibitory effect on rhizobia, even at high concentrations (Tu, 1981a; Heinonen-Tanski et al., 1982) and no effect on the non-symbiotic nitrogen fixers *Azotobacter* and *Beijerinckia* when used at 50 μg/ml (Ayanaba, 1981). It would be relevant to investigate the effects of these three fungicides in field studies at appropriate doses.

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