VOLATILE PHYTOTOXIC SUBSTANCES FORMED BY LITTER OF *PINUS RADIATA*

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ABSTRACT

Volatiles from radiata forest litter were shown to inhibit seedling growth of **Trifolium repens** L. (white clover), **Lolium perenne** L. (perennial ryegrass), and **Pinus radiata** D. Don. Seed germination of ryegrass and radiata was also reduced. This effect was not due to CO_2, reduced O_2 concentrations, or a compound soluble in water or paraffin wax. The authors have not yet shown that this effect occurs in the field.

INTRODUCTION

Pinus radiata D. Don is important economically in New Zealand not only as a forest crop but also when grown in association with agriculture. Under pure stands of radiata in Canterbury a thick layer of litter accumulates and the growth of seedlings of radiata and other species is sparse or absent. Because the lack of undergrowth might in part be due to allelopathy it was thought worthwhile to investigate the possible effects of radiata litter on the growth of seedlings of radiata and other plants.

There have been reports of the production of volatile metabolites by fungal cultures (Hutchinson, 1973), and because radiata litter supports vigorous fungal growth the effect of volatile substances evolved from the litter on seed germination and seedling growth was investigated.

MATERIALS AND METHODS

Basic Experimental Procedure

Litter was collected from beneath a mature stand of *Pinus radiata* (39-yr old, 300 stems/ha) at Ashley Forest, Canterbury. Despite moist site conditions undergrowth was sparse, and there were very few radiata seedlings even though numerous seed wings were seen in the litter.

In the first set of experiments cores (14 cm diam.) of the litter horizon, which ranged from 1.0 to 14.8 cm deep, were removed from positions determined by random

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co-ordinates from within the sampling area (100 m^2). Twenty litter cores were placed in 18-litre tins fitted with inlet and outlet tubes (Fig. 1). In later experiments 40 cores of litter cut with a 10 cm diam. steel corer were placed in each tin.

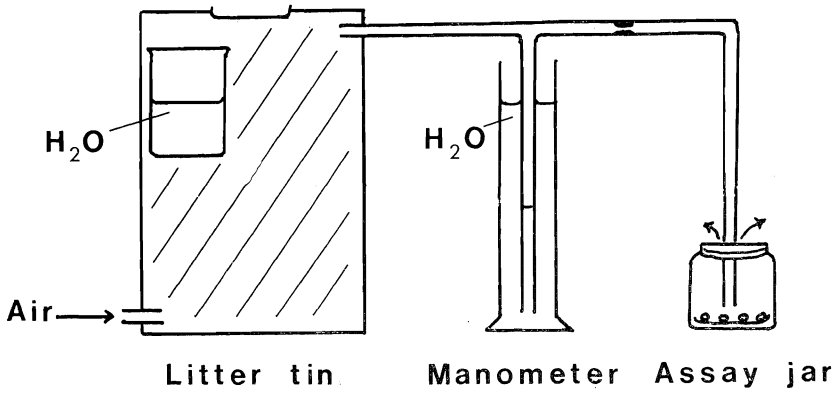

FIG. 1—Apparatus used for assaying litter volatiles

After placing a beaker containing 200 ml H_20 in each tin, the tins were sealed and incubated at 25°C for 4 days before the tin atmosphere was assayed. Control tins containing no litter were treated identically.

Preserving jars (500 ml "Agee" Utility) with a hole (8 mm diam.) in the metal seal were used as assay jars. Seedlings or seeds were placed on damp Whatmans seed test paper in each jar. Litter vapour for assay was displaced from the litter tins by compressed air and conducted by glass tubing into the assay jars (Fig. 1). A manometer was used to regulate the flow rate so that 1 litre of the litter atmosphere was passed into each assay jar. A loose fit of the glass tube in the lid of the assay jar permitted flushing of the jar. After treatment the holes were sealed with corks covered with polythene film. The jars were incubated in the dark at 25°C.

For seedling assays white clover and perennial ryegrass seed (supplied by Grasslands Division, D.S.I.R., Lincoln) were surface sterilised in 14% (v/v) "Janola" (NaOCl) for 5 min, rinsed with sterile distilled H_2O and germinated on moist sterile filter paper in the dark at 25°C. Radiata seed (supplied by Forest Research Institute, Rotorua) was washed in running tap water for 24 hours, stratified at 3°C for 24 hours, surface sterilised in 100 vol. H_2O_2 for 20 min., and germinated in sterile vermiculite at 25°C. Day-old clover, 2-days-old ryegrass and 5-days-old radiata seedlings were selected for assay. The assay jars were incubated for 4 days (clover and ryegrass) or 7 days (radiata). After incubation the hypocotyl length of clover and radiata seedlings, and the plumule length of ryegrass seedlings (combined length of the mesocotyl, coleoptile, and the first leaf) were measured to the nearest 1 mm.

Conditions for the seed germination tests, in which the same seed stocks were used, are given in section (b) below.

Using the basic procedures described above the following were investigated:

(a) *Effects of litter volatiles on seedling growth*

Volatiles in the atmospheres of six tins of litter (20 cores/tin) and two control tins

after 4 days' incubation were assayed with clover and ryegrass seedlings. Five jars of seedlings of each species per tin were treated consecutively. The litter volatiles were reassayed with clover after a further 7 days' incubation.

With a second collection of litter (20 cores/tin) one group of three tins with litter and one control tin was assayed with clover and ryegrass seedlings, and a second group with clover and radiata.

(b) *Effects of litter volatiles on seed germination*

Using the first collection of litter the effect of litter volatiles on the germination of surface-sterile seed of ryegrass and clover was investigated. Assay jars containing 300 seeds of ryegrass or clover were treated with vapour. Each tin was assayed with one jar of clover and another of ryegrass so that for each species there were two control and six experimental jars. Germination percentages were determined after 3 and 4 days' incubation at 25°C for clover and ryegrass respectively. A seed was classed as germinated if the radicle protruded through the seed coat.

An assay using the same procedures was made on the second collection of litter but using ryegrass (2 jars with 200 seeds/tin) and radiata (3 jars of 100 seeds/tin). Radiata germination was measured after 7 days' incubation.

(c) *CO_2 accumulation and O_2 depletion*

Litter vapour was assayed with white clover seedlings after CO_2 had been removed by passing the vapour through a gas washing bottle containing 125 ml 0.9 M NaOH placed in the outlet tubing assembly from the litter container between the tin and the manometer (see Fig. 1). Two tins, each containing 40 cores of litter, and one control tin were assayed with the CO_2 trap in place with 5 jars of white clover seedlings. The assay was repeated without the CO_2 trap. The NaOH was titrated with 1.25 M HCl to measure the CO_2 trapped from each tin.

Vapour obtained from a second group of two tins of litter and one control tin was replenished with O_2 to reduce or eliminate any effects of O_2 depletion by decomposing litter in the sealed tins on the growth of white clover seedlings in the assay jars. Any CO_2 formed in the tins was trapped in 200 ml 1.5 M NaOH held in beakers, replacing those containing water. Ten assay jars were treated per tin. Into five of these 50 ml O_2 was injected by hypodermic syringe to give a possible maximum and minimum of 28 and 10% respectively. Fifty ml N_2 was injected into each of the other five jars to act as controls. These jars could have contained a maximum and a minimum of 18 and 0% O_2 respectively. It was thus possible to detect (in the absence of CO_2) the effects of any O_2 deficiency.

(d) *Influence of paraffin wax and water traps on inhibitory effects of litter volatiles*

As monoterpenes have been shown to be involved in allelopathy (Muller, 1966) litter vapour was assayed after it had been passed over paraffin wax to trap any monoterpenes present. A gas washing bottle was packed with paraffin wax shavings and included in the gas line between the tin and the manometer (see Fig. 1).

To check the effectiveness of the trap, a small amount of camphor was placed in a

5-litre flask and left overnight to evaporate. When this vapour was passed through the trap three observers could not detect the characteristic odour of camphor though the smell was strong if the trap was removed.

One control and two litter tins (40 litter cores/tin) were assayed using white clover seedlings. Each tin was assayed with five jars with vapour which had passed through the trap and five with untreated vapour.

Because some volatile primary metabolites are soluble in water, e.g., ethanol, acetic acid and acetone, litter vapour from a second group of containers was assayed after passing through a gas washing bottle containing 125 ml H_2O.

RESULTS

(a) *Seedling growth*

In the experiments with the first two collections of litter, the presence of volatiles evolved from radiata litter was associated with a significant reduction in seedling growth (Table 1). With the first collection after 4 days' incubation there was significant variation in the inhibitory effect of litter volatiles from different containers on the growth of ryegrass and clover seedlings but such variation was not evident after 11 days' incubation in both the control and the litter vapour treatment. However, there was little change in the level of inhibition, which was 36.3% and 39.0% of control respectively for 4 and 11 days of litter incubation.

TABLE 1—The effect of vapour from incubated **P. radiata** litter on the growth of ryegrass, white clover and radiata seedlings (back-transformed means in mm*)

Litter collection	Time (days) of litter incubation	Species	Treatment		C.V. of transformed data
			Control	Litter vapour	
1st	4	Ryegrass	27.76[a]	20.58[b]	
	4	Clover	17.61[c]	6.40[e]	
	11	Clover	15.24[d]	5.94[f]	23%
2nd	4	Ryegrass	32.05[g]	21.71[h]	
	4	Clover	18.18[h]	5.86[j]	
	4	Radiata	21.96[h]	13.36[i]	28%

* Because of heterogeneity of variance, data was transformed using the log transformation before computing a nested analysis of variance. Group means of the transformed data were compared using the Student-Newman-Keul's test, and the results of this test are given in the table of back-transformed means. Means not significantly different at the 5% level have the same superscript. Each litter collection was analysed separately and no comparisons have been made between collections.

(b) *Seed germination*

With the first collection of litter (Table 2) the proportion of white clover seed that germinated was increased by litter volatiles. However, the volatiles reduced germination of both ryegrass and radiata seed. These effects probably indicate changes in germination rate rather than total germination.

TABLE 2—The effects of vapour from incubated **P. radiata** litter on the germination % of clover, ryegrass and radiata seed

Litter Collection	Time (days) of litter incubation	Species	Treatment		Probability of a greater X^2 value
			Control	Litter vapour	
1st	4	Clover	79.9	84.7	0.01
	4	Ryegrass	77.0	59.2	<0.001
2nd	11	Ryegrass	81.8	42.3	<0.001
	11	Radiata	40.6	16.1	<0.001

(c) *CO_2 and O_2 effects*

Litter volatiles adversely affected white clover seedling growth both in the presence (5.5%) and the absence of CO_2 (Table 3). Rather more inhibition occurred where CO_2 had been removed from the vapour, and it is possible that substances antagonistic to the inhibitor were removed in the trap.

TABLE 3—The response of clover hypocotyl growth to litter vapour after removal of CO_2, and water and wax soluble substances and after addition of O_2 (back-transformed means in mm*). Litter incubation time was 4 days

Litter collection	Treatment of vapour	Treatment		C.V. of transformed data
		Control	Litter vapour	
3rd	untreated	19.26^{a}	7.52^{b}	
	NaOH trap	19.52^{a}	5.51^{c}	22%
4th	50 ml O_2/jar	19.00^{d}	5.92^{e}	
	50 ml N_2/jar	20.63^{d}	6.53^{e}	14%
5th	untreated	19.04^{f}	7.14^{g}	
	water trap	18.44^{f}	6.47^{h}	
	wax trap	19.27^{f}	6.55^{h}	18%

* See Table 1.

(d) *Influence of paraffin wax and water traps*

The presence of these traps slightly increased the inhibitory effect of litter volatiles (Table 3). It may be that, as suggested in section (c) above, substances antagonistic to the inhibitor are removed in these traps. However, it is evident that the inhibitor is not absorbed effectively in either water or wax traps.

DISCUSSION

The experiments described above have demonstrated the presence of a volatile substance (or a mixture of volatile substances) evolved from incubated radiata litter that inhibits the growth of radiata, ryegrass and white clover seedlings and the germination of radiata and ryegrass seed, and stimulates the germination of clover seeds. The active substance has not been identified but it does not appear to be CO_2, a water-soluble metabolite, or a monoterpene. The experiments have not demonstrated whether the inhibitory volatile is evolved from *Pinus radiata* tissues *per se,* or if formed during their decomposition by litter-inhabiting organisms.

It is possible that the effects recorded in the laboratory result from substances produced in response to the mechanical disturbances of the litter during sampling, either through the release of additional substrates for microbial activity, or from a specific physiological reaction to damage.

We are endeavouring to identify the volatile compound and to determine if it occurs in significant quantities under field conditions.

ACKNOWLEDGMENTS

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