

ALLELOCHEMICALS FOR CONTROL OF PLANT-PARASITIC NEMATODES. 1. *IN VIVO* NEMATICIDAL EFFICACY OF THYMOL AND THYMOL/BENZALDEHYDE COMBINATIONS[†]

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ABSTRACT

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Greenhouse experiments were conducted to evaluate the nematicidal activity of thymol, a phenolic monoterpene present in the essential oils of several plant families. Thymol was added to soil at rates of 25-250 ppm. Initial and final population densities of *Meloidogyne arenaria*, *Heterodera glycines*, *Paratrichodorus minor*, and Dorylaimoid nematodes, as well as disease incidence, declined sharply with increasing dosages of thymol. Thymol was also applied at 0, 50, 100, and 150 ppm to soil in combination with 0, 50, and 100 ppm benzaldehyde, an aromatic aldehyde present in nature as a moiety of plant cyanogenic glucosides. Combinations in which benzaldehyde was applied at 100 ppm showed synergistic effects in suppressing initial and final soil populations of *M. arenaria* and *H. glycines*. Significant reductions in root galling and cyst formation on soybean were attributable to thymol at ≥ 50 ppm.

Key words: benzaldehyde, fumigants, *Glycine max*, *Heterodera glycines*, *Meloidogyne arenaria*, *Paratrichodorus minor*, natural products, nematicides, phenolics, root-knot nematode, soybean, soybean cyst nematode, terpenes, thymol.

RESUMEN

Soler-Serratos, A., N. Kokalis-Burelle, R. Rodríguez-Kábana, C. F. Weaver y P. S. King. 1995. Aleloquímicos para el control de nematodos fitoparásitos. *Nematropica* 26:57-71.

Se realizaron experimentos en invernadero a fin de evaluar la actividad nematicida del timol, un monoterpene fenólico presente en los aceites esenciales de diversas familias vegetales. El timol fue incorporado al suelo a razón de 25-250 ppm. Dosis crecientes de timol redujeron drásticamente las poblaciones iniciales y finales de *Meloidogyne arenaria*, *Heterodera glycines*, *Paratrichodorus minor* y nematodos Dorylaimidos, así como la incidencia de los endoparásitos. El timol fue aplicado al suelo en combinación con benzaldehído, un aldehído aromático presente en la naturaleza como subunidad de los glucósidos cianogénicos vegetales. El timol en concentraciones de 0, 50, 100, y 150 ppm fue combinado con benzaldehído a 0, 50, y 100 ppm. Las combinaciones en las que el benzaldehído fue aplicado a razón de 100 ppm mostraron efectos aditivos respecto a la reducción de las poblaciones iniciales y finales de *M. arenaria* y *H. glycines* en el suelo. Reducciones significativas en el número de agallas y quistes en plantas de soja fueron atribuidas al timol en concentraciones ≥ 50 ppm.

Palabras clave: benzaldehído, control químico, fenóles, fumigantes, *Glycine max*, *Heterodera glycines*, *Meloidogyne arenaria*, nematicidas, nematodo agallador, nematodo del quiste de la soja, productos naturales, soja, terpenos, timol.

[†]Portion of a M.S. thesis submitted by the first author to Auburn University.

INTRODUCTION

Environmental risks and food safety concerns from the use of many synthetic nematicides has resulted in a favorable climate for research involving alternative methods of nematode control in agronomic crops (Cook and Baker, 1983). Several researchers have suggested that naturally occurring biochemicals and plant allelochemicals, incorporated in integrated pest management (IPM) systems, could achieve effective reductions in target phytopathogens while minimizing environmental risks (Ferguson and Alford, 1985; Hedin, 1991).

Thymol (isopropyl-*m*-cresol) is a volatile, phenolic monoterpene present in the essential oils of several plant families, widely distributed throughout the Mediterranean region and other temperate zones of the world (Baerheim Svendsen and Scheffer, 1985; Brunke, 1986). Generally prepared from the oil of thyme (*Thymus vulgaris* L.), thymol is used in human and veterinary medicine for its antiseptic, antifungal, and anthelmintic properties (Wilson *et al.*, 1977). It is also used by food and fragrance industries for its pleasant, spicy-herbal aroma (Bauer *et al.*, 1990). Benzaldehyde (benzoic aldehyde) is a volatile, colorless liquid with a pungent aroma reminiscent of bitter almonds. It is found in nature in several cyanogenic glucosides (Harborne and Baxter, 1993). The fungicidal and fungistatic properties of benzaldehyde have long been recognized (Flor, 1926).

There are several reasons to explore the potential of applying mixtures of allelochemicals to soil for control of soilborne pathogens. One of them relates to economic feasibility, which is an important limiting factor to the introduction of allelochemicals in agriculture (Ferguson and Alford, 1985; Ku, 1987). Costs might be

reduced by combining readily available compounds, i.e. cheap by-products of various industrial processes with more active, expensive allelochemicals. Biochemical interactions among plants and microorganisms frequently involve the combined action of 2 or more compounds showing synergistic or additive effects on the target organisms (Einhellig, 1987).

This research was conducted as part of an ongoing study on the value of naturally occurring compounds as nematicides (Soler *et al.*, 1992). The objectives of this study were to determine the *in vivo* nematocidal activity of thymol, alone or in combination with benzaldehyde, and to evaluate the effects of these allelochemicals on disease incidence, plant growth, and soil enzymatic activity used as an indicator of soil microbial activity.

MATERIALS AND METHODS

Soil samples were collected from a soybean (*Glycine max* L.) field near Elberta, Alabama, U.S.A., which was naturally infested with a mixture of *Meloidogyne arenaria* and *Heterodera glycines*. The soil was a Norfolk sandy loam (fine loamy, siliceous thermic, Typic Paleudults, pH 5.3, <1.0% organic matter). Soil was screened (1 mm mesh) and mixed 1:1 with coarse builder's sand. This mixture (referred to hereafter as soil) was apportioned in 1.2 kg quantities in 4 L capacity polyethylene bags. In Experiment I, a stock solution was produced by diluting 25 g thymol (T) in 100 ml ethanol. After performing the appropriate dilutions, 1 ml aliquots were added to the soil to produce concentrations of 0, 25, 50, 75, 100, 125, 150, 200, and 250 mg thymol per kg soil (ppm). Experiment II utilized a 4 × 3 factorial design, in which thymol applied at rates of 0, 50, 100, and 150 ppm was combined with benzaldehyde (B) at rates of 0, 50, and 100 ppm. All

experimental units, including controls, received 1 ml ethanol as solvent. After thorough mixing, the treated soils of both experiments were transferred to 1 L, 10-cm-diam cylindrical plastic pots, and maintained moist in a greenhouse for 2 weeks. Subsequently, 200-cm³ soil samples were taken from each pot, and 100 cm³ of this soil were used to estimate preplant nematode populations by a modified Baermann technique (Rodríguez-Kábana and Pope, 1981). The remaining 100 cm³ of soil were air-dried (25°C), placed in polyethylene bags and stored in the dark at -4°C until analyzed for pH and soil enzymatic activity, used as a measurement of total biological activity.

Each pot was then planted with 5 'Davis' soybean seed. Plants were allowed to grow for 8 weeks, after which they were carefully removed from the soil. Shoot length and fresh weight of shoots and roots were determined. Other plant variables evaluated included the number of galls and cysts per g of root (calculated using the entire root system), and a disease index based on a 1 to 5 scale (subjective rating of the degree of root-rotting: 1 = lowest rot incidence; 5 = highest rot incidence). Populations of nematodes from the roots were determined by placing the entire root system in a screen and extracting the nematodes using the modified Baermann technique. The number of nematodes per g of root tissue was then calculated. Final soil samples (100 cm³) were dried and stored as for preplant samples.

The pH was determined for preplant and final soil samples. Soil subsamples of 10 g were placed in 100 cm³ capacity plastic cups, and 10 ml of demineralized water were added. After mixing, the cups were allowed to stand at 25°C for 30 min. The pH of the suspension was then determined

electrometrically using a Corning Model 12 pH meter.

The rate of hydrolysis of fluorescein diacetate (FDA) is indicative of total biological activity in soils (Schnurer and Ross-wall, 1982). Hydrolysis of FDA by a variety of esterases yields fluorescein. Quantification of esterase activity was determined by spectrophotometry using a modification of the method described by Inbar *et al.* (1991). Soil samples of 2 g each were placed in 30 ml capacity plastic cups, and 10 ml phosphate buffer (pH 7.6) were added. The reaction was started by adding 1 ml FDA (Sigma Chemical Co., St. Louis, MO) dissolved in acetone (1 mg/ml). One ml acetone was added to each sample as a control. The extent of chemical degradation of FDA was assessed using 3 cups containing double-autoclaved soil. All the soil suspensions were then gently agitated, sealed, and placed in an incubator at 25°C for 3 hr, after which 10 ml acetone was added to stop the reaction. The suspensions were centrifuged at 4000 g for 10 min, and the optical absorbance of the supernatants was determined at a wavelength of 490 nm using a Milton Roy Co. Spectronic 601 spectrophotometer. A standard curve was prepared with solutions of fluorescein in acetone to cover the range from 0 to 200 µg/ml. Enzymatic activity was expressed as µg fluorescein released per hr per g of soil.

Experiments utilized randomized complete blocks with 8 replications. Nematological data was log-transformed prior to analysis (Noe, 1985). Logistic dose-response curves were fitted to data in Experiment I to obtain LC50 and LC90 values (Jandel Scientific Table Curve 3.10, AINS Software, NY). Standard procedures for analysis of variance and correlation analysis at $P \leq 0.05$ were used on both experiments unless otherwise stated (Steel and Torrie, 1980).

Table 1. Experiment I: Effects of thymol on nematode populations in soil with estimated LC50 and LC90 values from preplant sampling.

Nematodes	LC50(ppm) ^y	LC90 (ppm)	r ^z
<i>M. arenaria</i>	85	161	0.60*** ^z
<i>H. glycines</i>	145	201	0.66**
Non-parasitic	196	225	0.76**

^yLC values calculated after fitting logistic dose-response curves to log (P) plotted against log (dose). There were 8 replicates per treatment.

^zAstericks denotes (***) P ≤ 0.01 for fitted regression.

RESULTS

Preplant samples: In experiment I, preplant numbers of *M. arenaria*, *H. glycines*, and non-parasitic nematodes in soil declined with increasing dosages of thymol, although each nematode group responded differently to the compound (Table 1). According to estimated LC values, thymol was more toxic to *M. arenaria* than to *H. glycines* or non-parasitic nematodes. Soil esterase activity decreased steadily with increasing dosages of thymol (Fig. 1). Thymol at ≥50 ppm caused a consistent increase in soil pH of around 0.3 pH units (Table 2). Numbers of all nematodes were positively correlated with soil esterase activity, whereas *M. arenaria* populations were negatively correlated with pH.

Final samples: In experiment I, plant weights were slightly affected by thymol treatments in the range 25-150 ppm (Table 2). Plants treated with 250 ppm produced lowest top and root weights. Final soil and root populations of *M. arenaria* and *H. glycines* decreased with increasing rates of thymol, as did soil populations of *Paratrichodorus minor* and Dorylaimoid nematodes (Table 3). Calculated LC values for *M. arenaria* in final samples were lower than for preplant samples, whereas final LC values for *H. glycines* were almost identical to those at preplant. Thymol caused

marked reductions in numbers of root galls and cysts in roots with LC90 values (i.e. rates needed to cause a 90% reduction in incidence) of 106 and 144 ppm, respectively (Table 3). Final soil and root populations of non-parasitic nematodes were unaffected by thymol treatments. The disease index decreased linearly with increasing thymol in the range 0-200 ppm (Fig. 1) but increased to 2.4 at 250 ppm. Disease index was positively correlated with preplant esterase activity and with total populations of *M. arenaria* at both sampling times. Final soil pH was higher in treatments 200 and 250 ppm (Table 2). Densities of non-parasitic nematodes from soil and roots were positively correlated with pH. Soil and root population densities of all phytoparasitic nematodes were negatively correlated with pH.

Preplant samples: In experiment II, there were no significant interactions between thymol and benzaldehyde with regard to pH or populations of non-parasitic nematodes. Separately, the compounds caused significant increases in pH (T = 100, 150; B = 50, 100) and reduced populations of non-parasitic nematodes (T = 50, 100, 150, B = 100). For all other variables, the interactions between thymol and benzaldehyde were significant. Esterase activity was reduced by thymol alone (T = 100, 150) and benzaldehyde (B = 100) (Fig. 2a). In

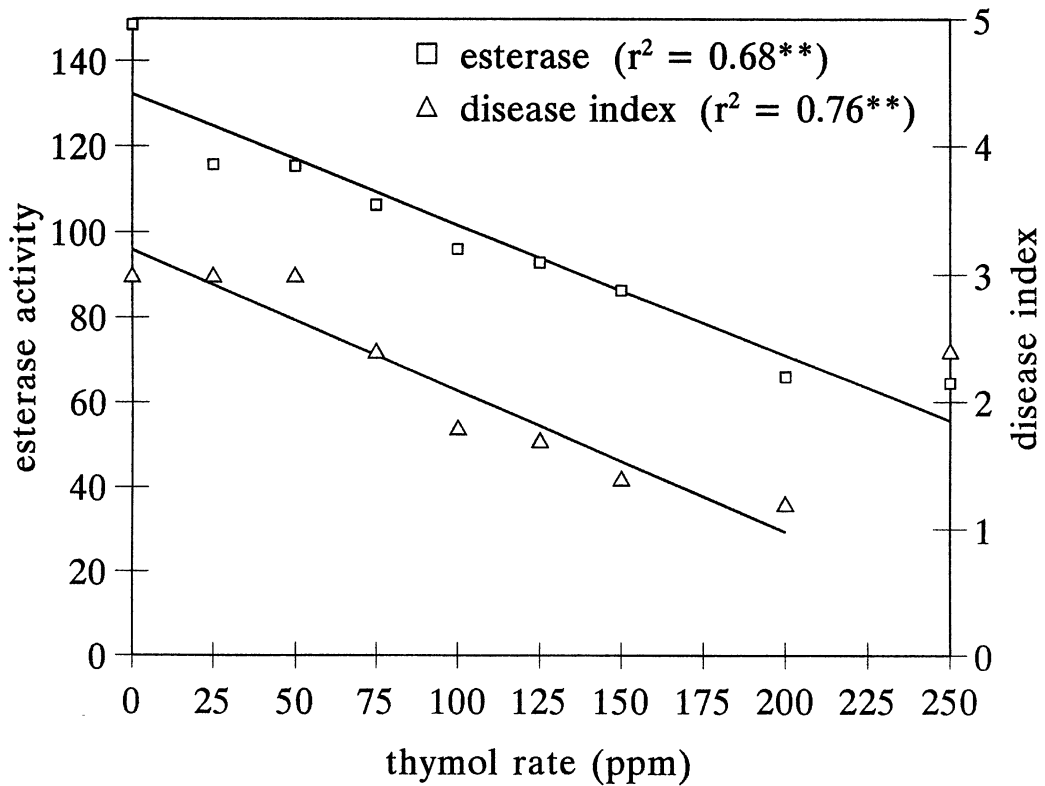


Fig. 1. Experiment I: Effects of thymol on preplant esterase activity and final disease index (subjective rating of the degree of root-rotting: 1 = lowest rot incidence, 5 = highest rot incidence). Data points are means of 8 replicates.

soil treated with benzaldehyde at 100 ppm, thymol failed to cause further reductions in esterase activity (Fig. 2a), and thus, no additive interaction between the factors could be detected. Indeed, benzaldehyde applications resulted in increased esterase activity in soil treated with 150 ppm thymol:

Thymol and benzaldehyde showed synergistic effects in reducing preplant populations of *M. arenaria* and Dorylaimoid nematodes (Figs. 3a and 5a) as seen by comparing the responses to thymol in treatments B = 0 with those in treatments B = 100. Thymol alone reduced preplant populations of *H. glycines* only at 50 ppm, whereas thymol combined with 50 ppm benzaldehyde was ineffective in suppressing preplant populations of *H. glycines*

(Fig. 4a). Benzaldehyde alone (treatments T = 0) reduced only preplant populations of Dorylaimoid nematodes (Fig. 5a).

Final samples: Soil pH was not affected by benzaldehyde but increased in response to thymol applications (T = 100, 150). Esterase activity was reduced by thymol/benzaldehyde combinations and by thymol alone (Fig. 2b) but not by benzaldehyde alone.

Populations of *M. arenaria* in roots were reduced synergistically by thymol/benzaldehyde combinations (Fig. 3b). By themselves, thymol and benzaldehyde did not affect soil or root populations of *M. arenaria*, but application of 100 ppm benzaldehyde to soil treated with thymol resulted in reduced numbers of *M. arenaria*. A similar

Table 2. Experiment I: Effects of thymol on soil pH and fresh weights of soybean plants.

Dose (ppm)	pH ^x		Plant weights (g)	
	Preplant	Final	Tops	Roots
0	5.29 ^y	5.76	3.65	3.47
25	5.33	5.66	3.11	3.28
50	5.50 ^{*z}	5.64	3.05	3.57
75	5.56 [*]	5.65	3.32	3.90
100	5.61 [*]	5.66	3.40	3.48
125	5.55 [*]	5.74	2.71	2.71
150	5.61 [*]	5.81	2.59	2.27 [*]
200	5.49 [*]	6.05 [*]	2.43 [*]	2.56
250	5.56 [*]	6.10 [*]	1.44 [*]	1.27 [*]

^xRegression could not be fitted satisfactorily for these data. Plant weight vs dose: $r = -0.90$ ($P \leq 0.001$). Root weight vs dose: $r = -0.86$ ($P \leq 0.01$).

^yData are means of 8 replicates.

^zAsterick denotes (*) Denotes significant differences from the control at $P \leq 0.05$ (FLSD).

trend was evident for *H. glycines* in soil but not in roots (Fig. 4). Synergistic effects also were observed in the decline of populations of Dorylaimoid nematodes (Fig. 5b). However, *dorylaimoid* populations were reduced only at the 150 ppm thymol alone, and numbers of these nematodes

were higher in soil treated with benzaldehyde alone. Populations of non-parasitic nematodes were higher in soil treated with benzaldehyde alone, whereas no significant effects could be detected in soil treated with thymol alone. Population levels reached by non-parasitic nematodes in

Table 3. Experiment I: Effects of thymol on soil and root populations of plant-parasitic nematodes with estimated LC50 and LC90 values from final sampling.

Nematodes	LC50 (ppm) ^{x,y}	LC90 (ppm) ^{x,y}	r ²
<i>M. arenaria</i> (soil + root)	65	85	0.82 ^{**z}
<i>H. glycines</i> (soil + root)	145	192	0.85 ^{**}
<i>P. minor</i> (soil)	77	100	0.77 ^{**}
Dorylaimoid (soil)	46	89	0.55 ^{**}
Galls/g root	30	106	0.60 ^{**}
Cysts/g root	<25	144	0.65 ^{**}

^xLC values calculated after fitting logistic dose-response curves to log (P) plotted against log(dose). There were 8 replicates per treatment.

^yRates needed to cause 50% or 90% reduction in numbers of *M. arenaria* induced galls or *H. glycines* cysts.

^zAsterick denotes (**) $P \leq 0.01$ for fitted regression.

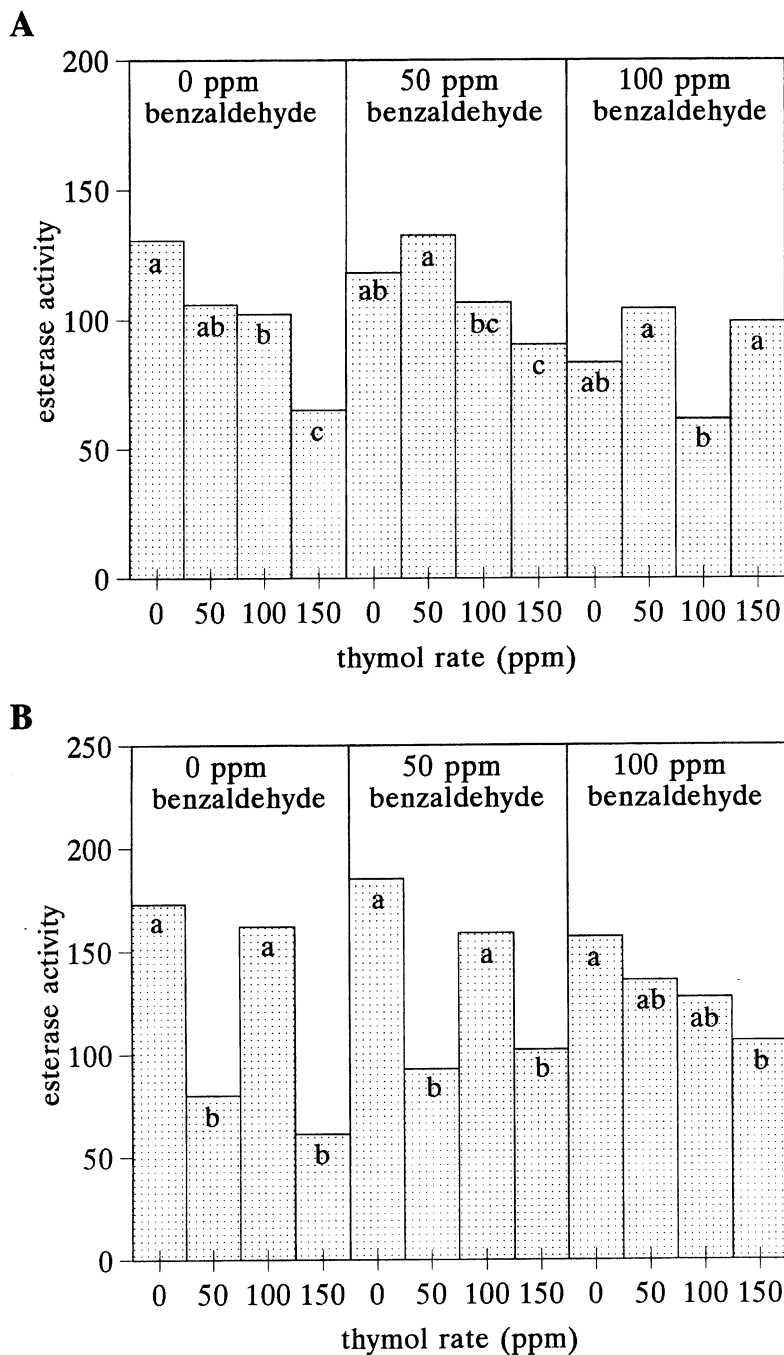


Fig. 2. Experiment II: Effects of combinations thymol/benzaldehyde on soil esterase activity. A) Preplant sampling. B) Final sampling. Within each benzaldehyde rate, letters denote significant differences at $P \leq 0.05$ (FLSD). Data are means of 8 replicates.

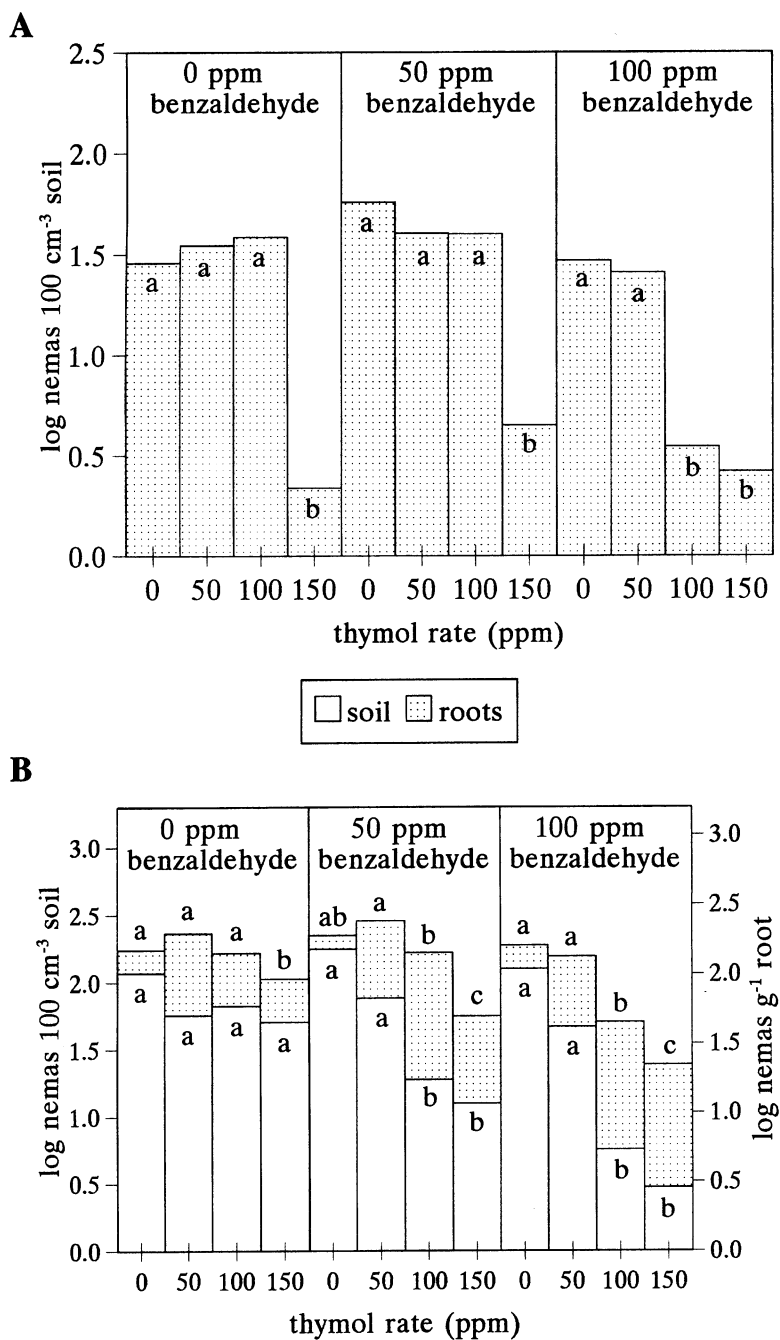


Fig. 3. Experiment II: Effects of combinations thymol/benzaldehyde on populations of *Meloidogyne arenaria*. A) Preplant sampling. B) Final sampling. Within each benzaldehyde rate, letters denote significant differences at $P \leq 0.05$ (FLSD). Data are means of 8 replicates.

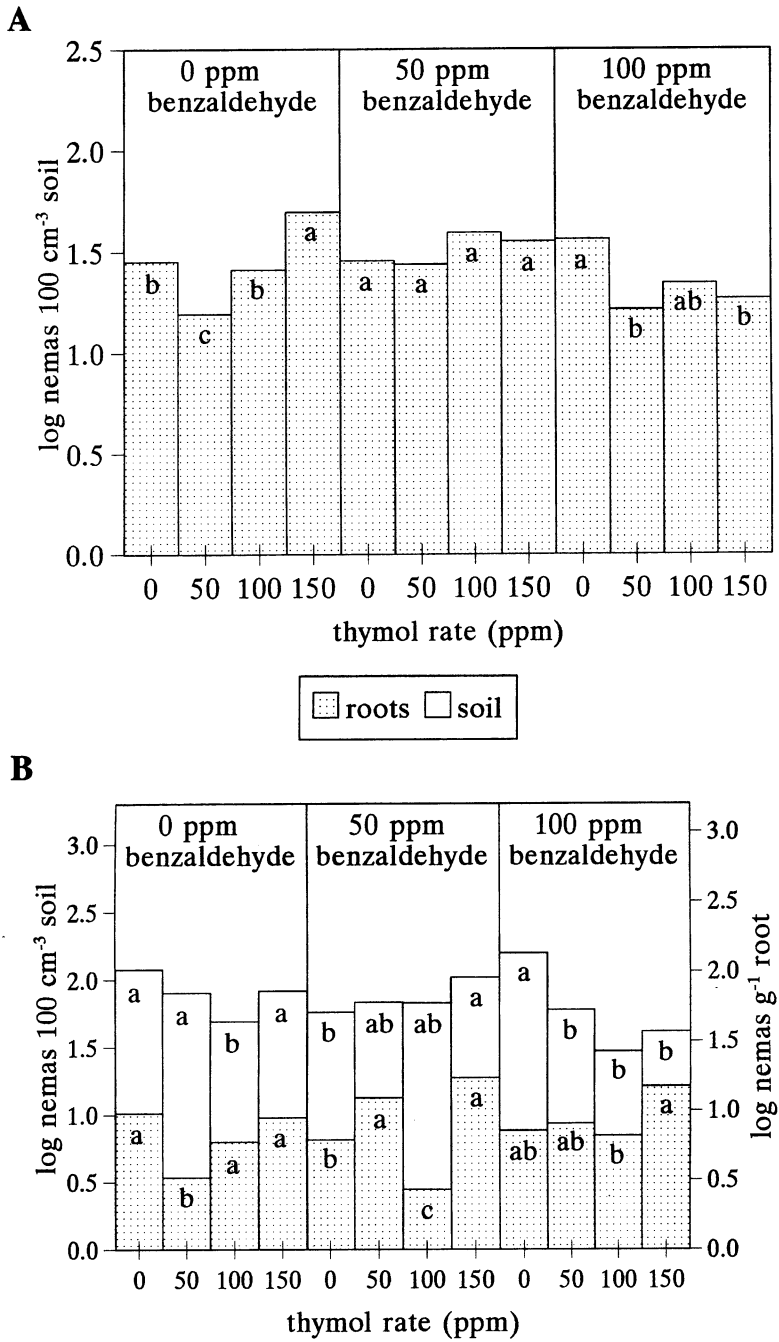


Fig. 4. Experiment II: Effects of combinations thymol/benzaldehyde on populations of *Heterodera glycines*. A) Pre-plant sampling. B) Final sampling. Within each benzaldehyde rate, letters denote significant differences at $P \leq 0.05$ (FLSD). Data are means of 8 replicates.

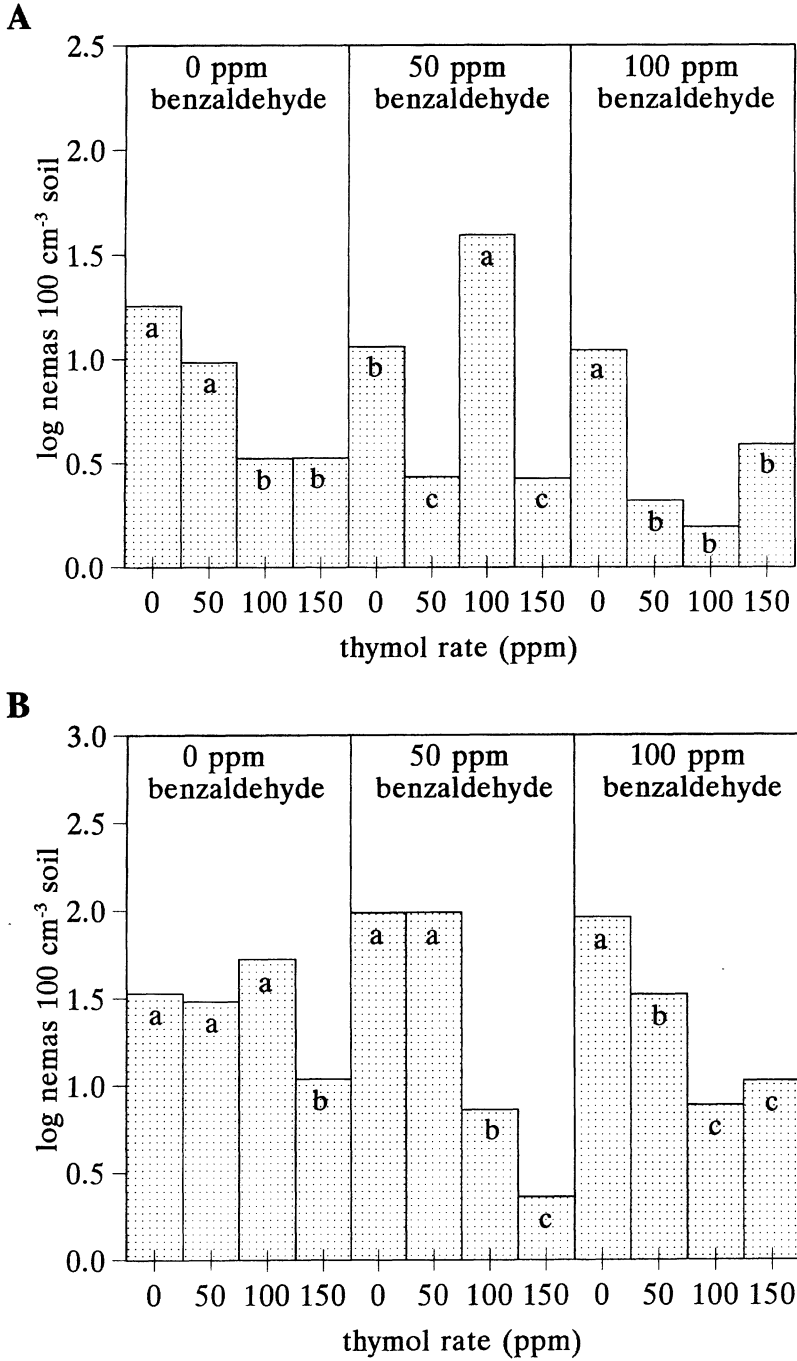


Fig. 5. Experiment II: Effects of combinations thymol/benzaldehyde on populations of Dorylaimoid nematodes. A) Preplant sampling. B) Final sampling. Within each benzaldehyde rate, letters denote significant differences at $P \leq 0.05$ (FLSD). Data are means of 8 replicates.

soil and roots at the end of the experiment were very similar among treatments (data not shown).

No significant effects could be attributed to thymol, benzaldehyde, or combinations of the compounds in regard to plant shoot lengths, weights, or root weights (data not shown). Root galling and cyst formation were substantially lower at all levels of thymol (Fig. 6a). The effects of benzaldehyde (alone or combined with thymol) for these 2 variables were negligible. However, the compounds showed synergistic effects in reducing disease index of roots (Fig. 6b).

DISCUSSION

Thymol and benzaldehyde exhibited wide-spectrum nematocidal activity. Populations of *M. arenaria* and Dorylaimoid nematodes appeared to be more sensitive to the allelochemicals than *H. glycines* or non-parasitic nematodes. The similarities between the functions describing the decline in phytoparasitic nematodes at both sampling times suggest that the compounds acted as nematocides or ovicides rather than nematostatics. In Experiment I, the pattern of esterase activity seems to corroborate this view, which is in accordance with the irreversible action of thymol on living cells (Wilson *et al.*, 1977). Thus, esterase activity could be a good estimator of the efficacy of thymol for controlling some phytoparasitic nematodes. In contrast, esterase activity tended to be higher in soils treated with thymol/benzaldehyde combinations than in treatments with thymol alone. Utilization of benzaldehyde as a nutritional source by microorganisms tolerant of thymol may explain this effect. Induction of substrate cometabolism (*e.g.* via inducible mixed-function oxidases) may hasten the microbial degradation of both compounds when applied

simultaneously (Schoknecht and Otto, 1989; Slater and Lovatt, 1984). An alternative explanation could involve the formation of less biocidal condensation products (triphenylmethane derivatives) from thymol and benzaldehyde through the Cannizzaro reaction, which is a common reaction of benzaldehyde in the presence of phenolics (Babor and Ibarz, 1979).

In Experiment I, application of thymol resulted in reduced plant top weights. Furthermore at 250 ppm, the tendency of lowered disease index was reversed, probably due to phytotoxic effects. This is indicative of a narrow therapeutic ratio between phytotoxic threshold and nematocidal threshold. Nevertheless, this ratio could be widened by a further delay in planting.

When comparing preplant populations of phytoparasitic nematodes with disease variables in both experiments, other factors besides direct toxicity of the allelochemicals seemed to account for the sharp declines in disease incidence. Three non-exclusive explanations may be suggested: (i) stimulation of a beneficial microflora by the compounds or the products of their degradation; (ii) altered host response; and (iii) establishment of a physico-chemical environment deleterious to phytonematodes. Shifts in populations of microorganisms, mainly bacteria, have been observed following treatments with aromatic and heteroaromatic pesticides (Bollen, 1979). The 'partial sterilization effect' of fumigation, by which microorganisms surviving the treatment proliferate at the expense of dead cells, has been observed previously (Waksman, 1952). Increased bacterial numbers may account for the recovery in densities of non-parasitic nematodes, which feed largely on soil bacteria (Yeates, 1979). Thymol and benzaldehyde also may stimulate a particular microflora capable of inducing host resistance or promoting nematode antago-

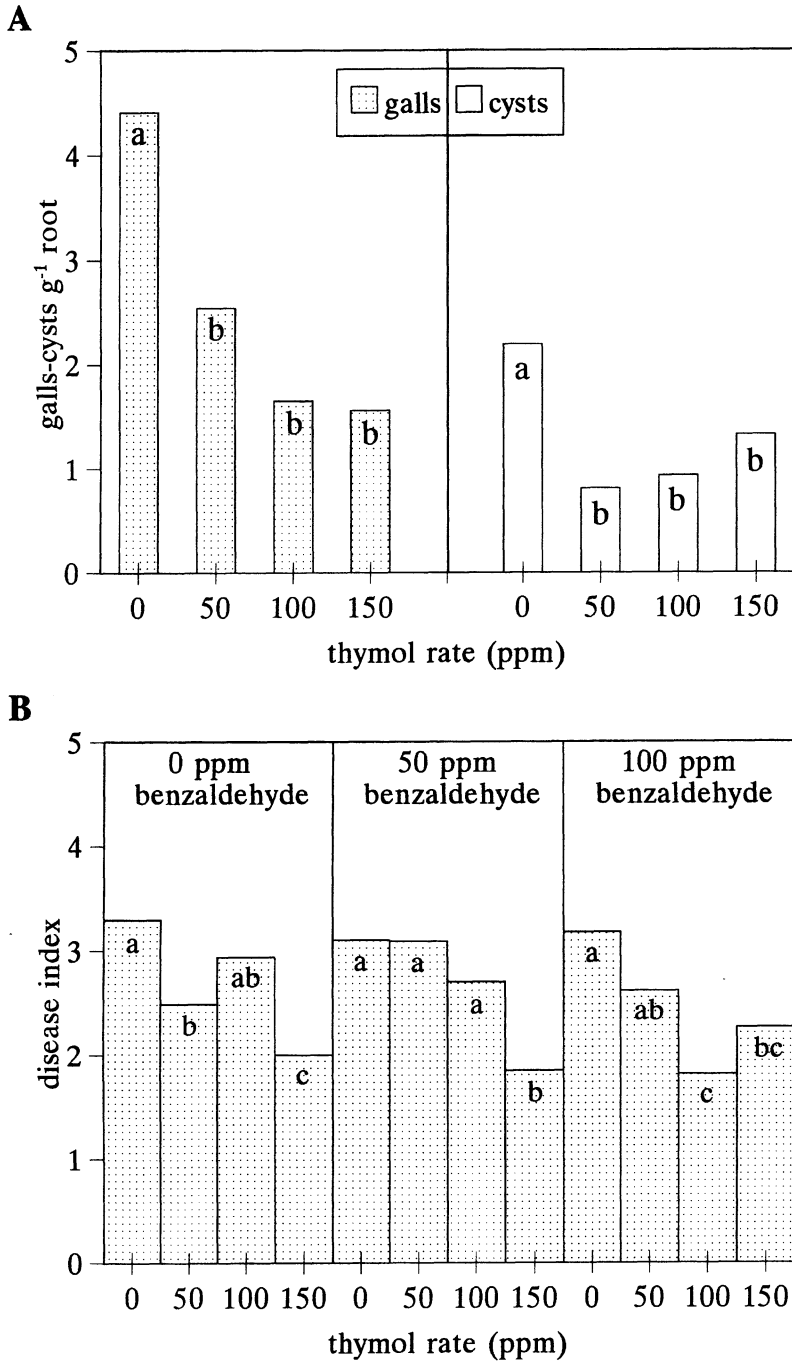


Fig. 6. Experiment II: Effects of combinations thymol/benzaldehyde on A) Root galling and cyst formation (interaction not significant, letters denote significant differences at $P \leq 0.05$); and B) disease index (subjective rating of the degree of root-rotting: 1 = lowest rot incidence; 5 = highest rot incidence). Within each benzaldehyde rate, letters denote significant differences at $P \leq 0.05$ (FLSD). Data are means of 8 replicates.

nism (Becker *et al.*, 1988; Kloepper *et al.*, 1992; Soler-Serratos *et al.*, 1994). It is well known that pesticides exert a selective pressure on soil microflora, often causing saprophytes to be favored over parasites (Bollen, 1979). Selection of a beneficial microflora by phenolic monoterpenes would explain the advantage conferred on plants in the Lamiaceae that produce those compounds (Katz *et al.*, 1987).

Altered host response due to the addition of thymol or benzaldehyde to soil might have been possible through the fixation of the allelochemicals to soil colloids without denaturation (Muller and Del Moral, 1966). Degradation products including phenolics or hydroquinones might have resulted in an altered host-nematode interaction (Alam *et al.*, 1980).

In Experiment I, shifts in soil pH were negatively correlated with soil and root populations of phytoparasitic nematodes. Increased availability of ammoniacal nitrogen ($\text{NH}_4^+\text{-N}$), as often observed following fumigation (Bollen, 1979; Sato, 1983; Waksman, 1952), could explain the shifts in soil pH with concomitant reductions in phytonematode populations due to ammonia toxicity (Rodríguez-Kábana, 1986). Waksman (1952) attributed the abundant liberation of ammonia following treatment with various volatile and non-volatile antiseptics to bacterial proliferation. Extensive metabolism of organic constituents made available to bacteria surviving the treatment would result in the liberation of large amounts of nitrogen as ammonia. This phenomenon, accompanied by the selective impact that antiseptics have on nitrifying organisms (i.e. bacteria driving the oxidation of ammonia to nitrites and nitrates), should result in accumulation of ammonia in soil. In a study evaluating the impact of pentachlorophenol (PCP) on soil microflora, Sato (1983) substantiated these premises by demonstrating that

increasing soil pH and bacterial populations were accompanied by strong depressions in the nitrification processes which resulted in $\text{NH}_4^+\text{-N}$ accumulation.

The rates used in our experiments showing strong nematicidal activity (100-250 ppm) are roughly equivalent to 220-560 kg/ha, considering that the weight of a ha of soil to a 15-cm depth (cultivable zone) is 2.242×10^6 kg. These rates fall into the range of recommended dosages of several fumigant nematicides, i.e. 60-1,200 kg/ha for methyl bromide, methyl isothiocyanate and chloropicrin (Alabama Cooperative Extension Service, 1988). Therefore, thymol and benzaldehyde could compete with commercially available nematicides if the conditions for their bulk production and commercialization were acceptable. Optimization of application methods may improve their value as nematicides, and post-treatment plastic mulching is a possibility considering the volatility of these compounds.

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