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The Potential of Thiarubrine C as a Nematicidal Agent against Plant-parasitic Nematodes

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Abstract: Thiarubrine C, a polyacetylenic 1,2-dithiin isolated from the roots of *Rudbeckia hirta* (Asteraceae), exhibited strong nematicidal activity in in vitro and growth chamber assays. Thiarubrine C was toxic, in the absence of light, to the plant-parasitic nematodes *Meloidogyne incognita* and *Pratylenchus penetrans* at LC_{50}s of 12.4 ppm and 23.5 ppm, respectively. A minimum exposure time between 12 and 24 hours was the critical period for nematode mortality due to thiarubrine C. Although thiarubrine C was not totally dependent on light for toxicity, activity was enhanced in the presence of light, especially with the microbivorous nematode, *Teratorhabditis dentifera.* Upon exposure of *M. incognita* juveniles to 20 ppm thiarubrine C for 1 hour, infection of tomato plants was greatly reduced compared to untreated checks. Thiarubrine C was also effective in reducing plant infection when mixed with soil 24 hours prior to or at planting, unlike other related compounds such as ∂-terthienyl.

Key words: assay, control, *Meloidogyne incognita,* nematicidal agent, plant extract, plant-parasitic nematodes, *Pratylenchus penetrans, Rudbeckia hirta, Teratorhabditis dentifera,* thiarubrine C.

Thiarubrines are red-colored 1,2-dithiins containing acetylenic moieties that are primarily present in the roots of species of the Asteraceae (Bohlmann and Zdero, 1985; Freeman et al., 1993). Rodriguez et al. (1985) and Towers et al. (1985) demonstrated the antibiotic properties of these compounds, showing that thiarubrine A was toxic to *Candida albicans* and *Saccharomyces cerevisiae* as well as the bacterial feeding nematode *Caenorhabditis elegans.* Similar results were reported with thiarubrine C (Constabel and Towers, 1989), and activity of both thiarubrine A and C was enhanced by exposure to UVA or visible light (Towers et al., 1985; Constabel and Towers, 1989). Thiarubrine C differs from thiarubrine A in that the double bond present in thiarubrine C is replaced by a triple bond in thiarubrine A (Fig. 1). However, thiarubrines appear to show considerable variability in biological activity depending upon the test organism. Gram positive bacteria appear to be more sensitive than gram negative bacteria; *Escherichia coli* was inhibited by thiarubrine A and thiarubrine C, while *Pseudomonas flavescens* showed no inhibition (Constabel and Towers, 1989). Hudson et al. (1986) found that only membrane-bound viruses were inhibited by thiarubrine A. Thiarubrine A and thiarubrine C exhibited more activity than thiarubrine D at equivalent doses against *Saccharomyces cerevisiae* (Constabel and Towers 1989), although thiarubrine C possessed greater toxicity under dark conditions against *S. cerevisiae* than did thiarubrine A.

Hijink and Suatmadji (1967) reported that *Rudbeckia hirta* L. (Asteraceae) suppressed root-parasitic nematodes in greenhouse pots, although the suppressive agent was not identified. Gommers and Voor in 't Holt (1976) observed that a large number of asteraceous plants that suppressed nematodes contained 1,2-dithiins in their roots. There are no reports describing the effects of thiarubrine C on plant-parasitic nematodes in terms of its toxicity or inhibition of plant infection. Our objective was to assess the toxic effects of thiarubrine C on *Meloidogyne incognita* (southern root-knot nematode), *Pratylenchus penetrans* (northern root-lesion nematode), and *Teratorhabditis dentifera* (bacteria-feeding nematode) and to assess the ability of thiarubrine C to inhibit plant infection by *M. incognita.*

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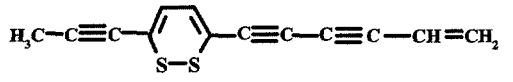
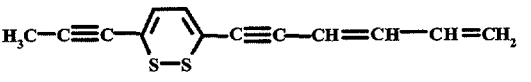


Thiarubrine A



Thiarubrine C

FIG. 1. Chemical structures of thiarubrine A and thiarubrine C.

MATERIALS AND METHODS

Isolation of thiarubrine C: Thiarubrine C was isolated from freeze-dried roots of wild *R. hirta* plants (Bohlmann and Kleine, 1965) collected from several locations in the Ithaca, New York (USA) area. Freeze-dried roots were extracted four times with 100% hexane (1 liter/100 g of roots) and then rotoevaporated *in vacuo* at low temperatures (37–40 °C) in dim light to minimize the breakdown of thiarubrine C. Samples were not evaporated to dryness in order to prevent polymerization of thiarubrines. Subsequently, two-column chromatography runs on silica gel 60 (particle size 0.063-0.200 mm, column dimensions 3 cm diam. × 27.5 cm long) were performed to separate thiarubrine C from other thiarubrines and thiophenes present. With hexane as the eluant, thiarubrine C eluted as the first red band, requiring approximately 220 ml solvent. A typical yield was 0.44 mg/g freeze-dried root.

High-pressure liquid chromatography (HPLC) was used to confirm the purity of thiarubrine C, using a Beckman (Fullerton, CA) HPLC system with 110 A pumps and a model 332 controller, coupled to a Waters (Milford, MA) Lambda-Max 480 LC spectrophotometer and a Hewlett-Packard (Wilmington, DE) 3390A integrator. Separations were performed with an Econosphere 5-µm C-18 reversed-phase column (5 mm × 250 mm long) (AllTech, Deerfield, IL) using isocratic elution with acetonitrile:water (60:40) at a flow rate of 1 ml/minute. Retention times were 8.13 and 8.48 minutes, respectively, for the Z and E isomers of thiarubrine C (Constabel et al., 1988).

Nematode cultures: Pratylenchus penetrans was obtained from a laboratory colony growing in alfalfa callus culture on White's medium (Riedel and Foster, 1970). *Meloidogyne incognita* eggs were obtained 6-8 weeks after inoculation of tomato plants (*Lycopersicon esculentum* cv. Rutgers). The root system was washed with cold tap water, cut into 1-cm pieces, covered with a solution of 20% bleach in tap water, and shaken for 4 minutes. Roots then were rinsed with tap water over 75-µm and 25-µm mesh sieves, and the eggs were retained on a 10-µm sieve. Eggs were transferred onto tissue paper, placed on a milk filter paper lying on a stainless steel grid in an aluminum pie-pan, then covered with deionized water. Every 2 days for up to 8 days, juveniles were collected by filtering the water through a sieve with 10-µm openings. *Teratorhabditis dentifera* (kindly provided by P. Timper) was maintained by weekly culture on *E. coli* lawns on nutrient agar plates.

Concentration response cureves: Both *M. incognita* and *P. penetrans* were used in this study. The assay was conducted in 24-well culture plates to which approximately 25 nematodes/well were added, with 6 replicates per treatment. Materials to be tested were dissolved in ethanol and diluted in deionized water to give the desired final concentrations (0, 5, 10, 20, 30, 50 ppm) with a final ethanol concentration not exceeding 1% in a total volume of 1 ml. Controls for this experiment consisted of 1% ethanol in deionized water and water alone. Plates were incubated in the dark at 20 °C, and non-motility and blackening of nematode interior as indicators of mortality were scored at 0, 24, 48, 72, and 96 hours, using an inverted microscope at a power magnification of ×40. Concentration response data were analyzed with a probit program, POLO-PC (LeOra Software, Berkeley, CA), and LC_{50} and LC_{90} (95% CL) were calculated for each nematode population. Each experiment was replicated at least twice.

Effect of time of exposure of thiarubrine C on nematode viability: To determine time of exposure to thiarubrine C required for mortality of *M. incognita,* 25 nematodes/well

were added into 24-well plates and treated with 20 ppm thiarubrine C at time zero as described above. At 4, 12, 24, or 48 hours, the aqueous layer containing thiarubrine C was removed and replaced with 1 ml of deionized water. Mortality was scored prior to rinsing the wells with deionized water and at the indicated time intervals after the aqueous layer had been replaced. Nematodes incubated continuously in the presence of 20 ppm thiarubrine C were used as the positive control, and nematodes incubated in 1% ethanol in deionized water were used as the negative control. Experiments were replicated at least twice.

Effect of light vs. dark on activity of thiarubrine C: Meloidogyne incognita and *T. dentifera* were added to two each separate 24-well plates at 25 nematodes/well. Six wells of each nematode were exposed to thiarubrine C at concentrations of 20 and 50 ppm; the negative control was 1% ethanol in deionized water. One plate of each nematode was exposed to a full spectrum light lamp of an intensity of 12.6 microeinsteins ($\mu E)/m^2$ s, at a distance of 25 cm, while one plate was kept in darkness at 18 °C. Non-motility was scored after 2, 8, and 24 hours of exposure. In conjunction with these experiments, thiarubrine C, at the concentrations mentioned above, was first exposed to light at the same intensity and distance until its typical redness was lost (typically 45 minutes), indicating conversion to thiophene C. This solution was then used to test both nematode species under lighted and dark conditions at 18 °C, as described above. Two independent replications of the experiment were carried out.

Effect of thiarubrine C on plant infectivity: To determine if thiarubrine C prevented *M. incognita* from infecting tomato plants, nematodes were exposed to 20 ppm thiarubrine C in 1% ethanol or incubated in 1% ethanol in deionized water for 0, 1, 4, 6, 8, or 24 hours. Plastic cone tubes, 4 cm diam. × 13.5 cm length, containing 3-week-old plantlets in autoclaved soil, were then infested with 500 nematodes/cone, with six replicates per treatment. Tubes were maintained in a growth chamber at 25 °C, with a light/dark cycle of 15:9 hours. Seven days after infestation, the roots were harvested and stained with acid fuchsin following the method described by Bridge et al. (1982).

To determine if thiarubrine C was effective against *M. incognitia* when mixed directly with soil, we used 6-well plates in which each well contained approximately 5 cm^3 of autoclaved soil mix. At the zero time point, approximately 300 nematodes per well in 1 ml of deionized water were added to the wells to which seedlings were to be added and 500 nematodes per well were added to the wells to which plantlets were to be added. Thiarubrine C, at a concentration of 50 ppm dissolved in 1 ml of 1% ethanol, was then applied to soil in those wells previously infested with *M. incognita*. Treatments employed both 2-week-old tomato plantlets and 2-day-old tomato seedlings, which were planted 1 cm deep, at the time of addition of thiarubrine C (0 hours) or 24 hours later. There were five replicates per treatment, and negative controls consisted of 1% ethanol in 1 ml of deionized water; experiments were independently replicated at least twice. The material was processed for analysis as previously described (Bridge et al., 1982).

Results

Concentration response curves: A concentration of approximately 15 ppm thiarubrine C killed 50% of the *M. incognita* juveniles, while 50 ppm thiarubrine C resulted in 100% mortality after 48 hours (Fig. 2A). At a thiarubrine concentration of 20 ppm, more than 80% of the juveniles were dead after 48 hours and, by 72 hours, 100% of the juveniles were dead. The effect of thiarubrine C on *P. penetrans* also was assessed (Fig. 2B). *Pratylenchus penetrans* was more resistant to 50 ppm thiarubrine C; only 50% of the nematodes were dead after 48 hours. At 96 hours of exposure, however, 100% mortality occurred. Table 1 gives the calculated LC_{50} and LC_{90} at 95% confidence limits for thiarubrine C on both *M. incognita* and *P. penetrans*; these values were determined at 48 hours and 72 hours, respectively.

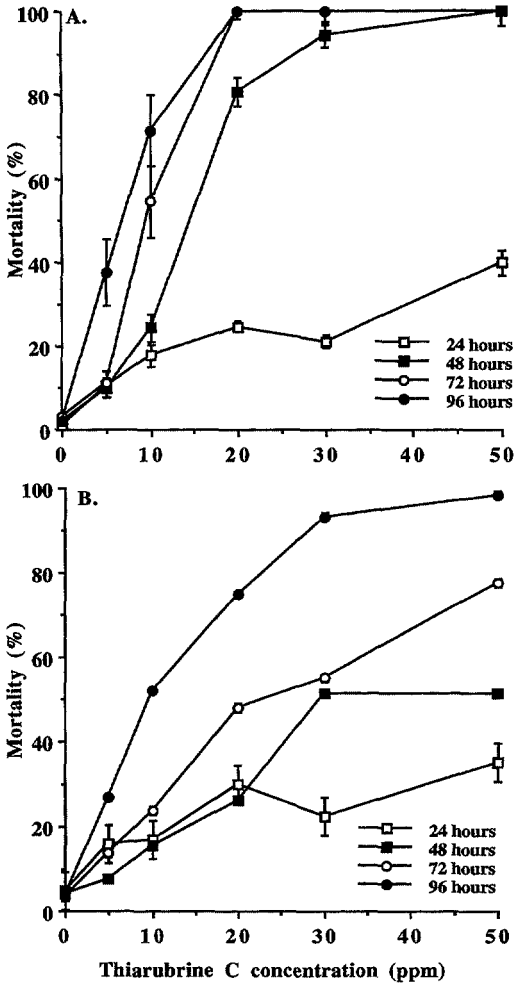


FIG. 2. Concentration response curves of mortality of *Meloidogyne incognita* (A) and *Pratylenchus penetrans* (B) after 24, 48, 72, and 96 hours of exposure to concentrations of thiarubrine C (0, 5, 10, 20, 30, and 50 ppm). The data points represent the means of the data. Bars represent standard errors of the means.

Effect of time of exposure of thiarubrine C on nematode mortality: Meloidogyne incognita was incubated in the presence of 20 ppm thiarubrine C for 4, 12, 24, or 48 hours, followed by removal of the aqueous layer containing thiarubrine C and replacement with water. After 4 hours and 12 hours exposure to thiarubrine followed by water only, *M. incognita* mortality was lower in washed wells than in those wells continuously exposed to thiarubrine C (Fig. 3). However, after 24 hours exposure to thiarubrine C at 20 ppm followed by water replacement, mortality continued to increase. The response curves were similar both for the nematodes that were continuously exposed to thiarubrine C and for those exposed for 24 hours and 48 hours followed by removal of the treatment.

Effect of light vs. dark on activity of thiarubrine C: After a 2-hour exposure to 50 ppm thiarubrine, *M. incognita* exhibited 15% mortality under lighted conditions and 5% mortality in the dark (Fig. 4A). After 24 hours under lighted conditions, 82% of *M. incognita* were dead at a thiarubrine concentration of 50 ppm (Fig. 4A), and 63% were dead at 20 ppm (Fig. 4B). Mortalities of *M. incognita* were fewer or lower after incubation in the dark for 24 hours, with 30% at 20 ppm and 45% at 50 ppm thiarubrine C. Thiophene C toxicity under light conditions was equal to or greater than that of thiarubrine C under dark conditions, but was less toxic than thiarubrine C under lighted conditions after 24 hours (Fig. 4A,B). After 2 hours exposure to thiarubrine C at both 20 or 50 ppm, 100% of *T. dentifera* were dead under lighted conditions, and 95% were dead when incubated in the dark (Fig. 4C,D). Thiophene C under dark conditions showed significant toxicity only to *T. dentifera* at 50 ppm but caused 100% mortality at both 20 and 50 ppm under lighted conditions. Control mortalities remained at baseline levels.

Effect of thiarubrine C on plant infectivity: Although exposure to a concentration of 50 ppm thiarubrine C for 48 hours was determined to be lethal for *M. incognita,* exposure to lower concentrations could affect the ability of nematodes to infect plants. Exposure of *M. incognita* to a concentration of 20 ppm thiarubrine C for up to 24 hours limited the infection of 3-week-old tomato seedlings. An exposure time of 1 hour reduced the number of nematodes that infected the roots of the host by 91% (Fig. 5). No infection of tomato seedlings was observed after *M. incognita* was exposed for 4, 6, 8, or 24 hours to 20 ppm thiarubrine C.

When 2-week-old tomato plantlets were transplanted into wells containing *M. incognita*-infested soil that had been treated 24

TABLE 1. LC_{50} and LC_{90} (expressed in ppm) of thiarubrine C on *Meloidogyne incognita* and *Pratylenchus penetrans*. CL = confidence limits.

Organism	Time (hours)	LC_{50} (95% CL) (ppm)	LC_{90} (95% CL) (ppm)
M. incognita	48	12.4 (8.6-17.6)	23.8 (16.9-55.7)
P. penetrans	72	23.5 (16.4-33.5)	112.7 (69.9-366)

hours previously with 50 ppm thiarubrine C, infection was reduced by 89% compared to untreated samples. The addition of thiarubrine at the time of transplanting (0 hours) reduced infection by approximately 59% (Table 2). In a similar experiment, where tomato seedlings were transplanted into *M. incognita*-infested soil treated for 24 hours with 50 ppm thiarubrine C or thiarubrine added at transplanting (0 hours), infection of tomato seedlings in treated soil was reduced by 94.4% after 1 week, compared with untreated soil. In all experiments, no phytotoxic effect of thiarubrine C was observed.

DISCUSSION

Thiarubrine C was an effective nematicidal agent against the plant-parasitic nematodes *M. incognita* and *P. penetrans*. We observed that *M. incognita*, a sedentary endoparasite, was more sensitive to the nematicidal activity of thiarubrines than *P. penetrans*, a migratory endoparasite. With *M. incognita*, thiarubrine concentrations of 30-50 ppm killed 90% or more of the juveniles after 48 hours exposure, whereas a concentration of 50 ppm killed only 50% of *P. penetrans* during the same time interval under dark conditions. The LC_{50} was greater for *P. penetrans* (23.5 ppm at an exposure time of 72 hours) than for *M. incognita* (12.4 ppm at an exposure time of 48 hours).

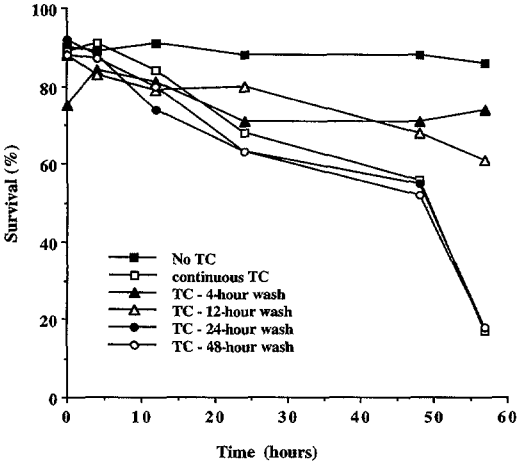


FIG. 3. Percentage of *Meloidogyne incognita* surviving after exposure to 20 ppm thiarubrine C, followed by replacement with water. Mortality was scored prior to rinsing the wells with deionized water, then scored at the indicated time intervals after the aqueous layer had been replaced. Nematodes incubated in 1% ethanol in deionized water were used as the negative control. TC = thiarubrine C.

Thiarubrine C compares favorably with other previously reported nematicides of natural origin. Terthienyl, a related sulfur-containing polyacetylene, is a potent nematicide; exposure to 1 ppm for 90 minutes caused 100% mortality of *Ditylenchus dipsaci* in vitro, but radiation was required to activate its nematicidal properties (Gommers, 1972). The steroid-glycoalkaloid, ∂-tomatine, exhibited an ED_{50} of 50 ppm against *Panagrellus redivivus;* the ED_{50} increased with decreasing pH (Allen and Feldmesser, 1970). Using an immersion test assay with the rice white-tip nematode *Aphelenchoides besseyi*, fatty acids from the roots of *Iris japonica*, such as undecanoic acid, gave 80-100% nematode mortality at 20 ppm after 24 hours (Munakata, 1983), and terpenoids from *Daphne odora* (Kogiso et al., 1976), such as odoracin, produced 100% mortality at 5 ppm after 5 days. A cyclic disulphide from the roots of *Asparagus officinalis*, asparagusic acid, was lethal to *M. hapla* at 55 ppm in water (Takasugi et al., 1975). A breakdown product of the allyl glucosinolate sinigrin, allyl isothiocyanate, had an LC_{50} of 40 ppm at 24 hours against *Caenorhabditis elegans*, a free-living nematode (Donkin et al., 1995). We found 100% mortality of the free-living

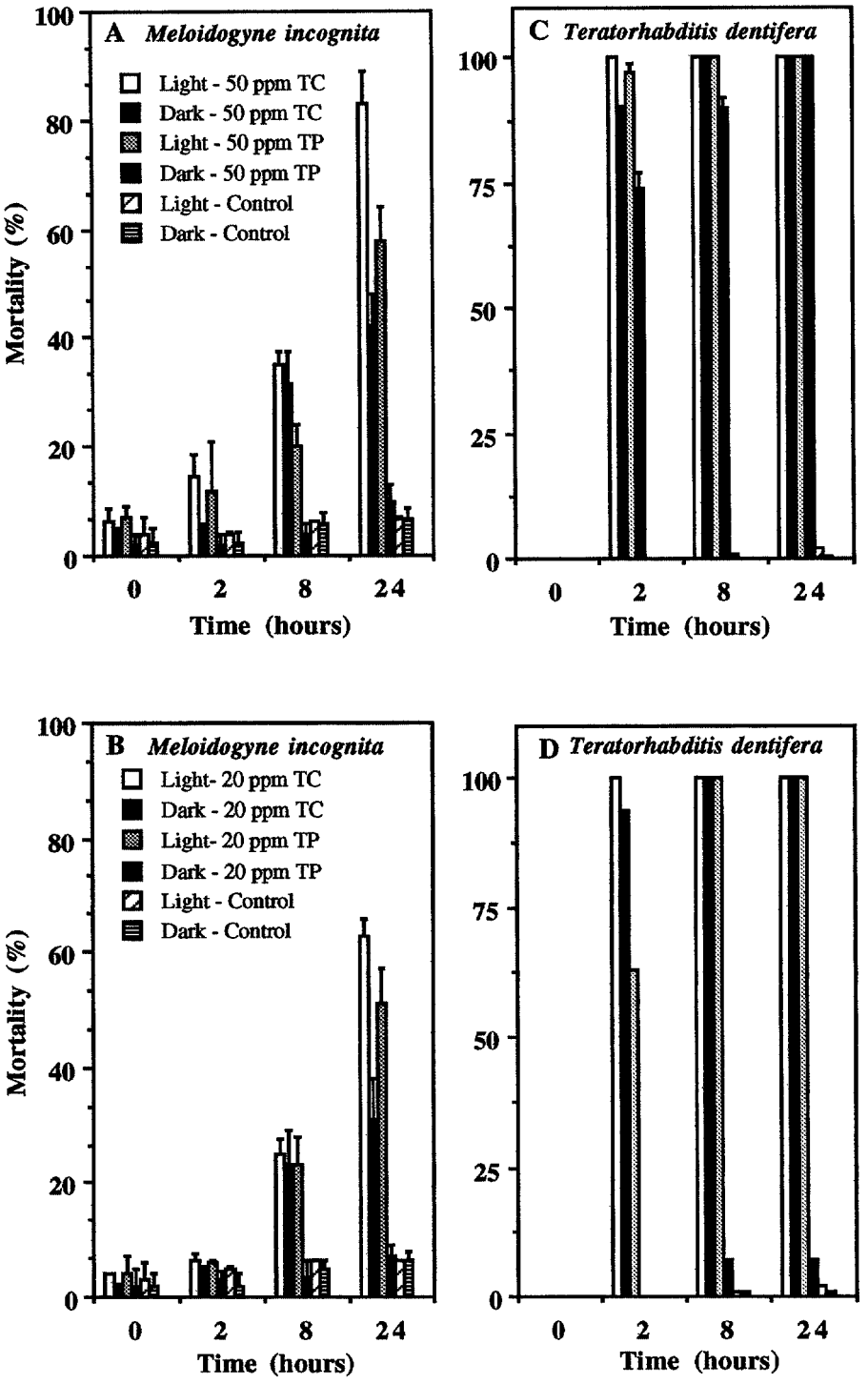


FIG. 4. Percent mortality of *Meloidogyne incognita* (A,B) and *Teratorhabditis dentifera* (C,D) at thiarubrine C (TC) or thiophene C (TP) concentrations of 50 ppm (A and C) and 20 ppm (B and D). Experiments were performed under dark and light conditions at 18°C. The data points represent the means (±SE) of two separate experiments (12 replicates per treatment). Bars are standard errors of the means. Legend in A also applies to B; legend in C also applies to D.

nematode *T. dentifera* after 8 hours at 20 ppm in the dark and within 2 hours under light, when exposed to thiarubrine C; longer exposure times were required for 100% mortality of *M. incognita.*

The requisite time of exposure to thiarubrine C could be a limiting factor to its usefulness as a nematicide. We found that a minimum time between 12-24 hours appeared to be the critical time point to cause mortality from exposure to 20 ppm of thiarubrine C. Recovery of nematodes has been studied after exposure to the synthetic nematicides, oxamyl and phenamiphos. *Ditylenchus dipsaci* recovered after exposure to 10,000 ppm oxamyl for 24 hours, whereas the effects of phenamiphos were reversible only for concentrations of up to 10 ppm (Bunt, 1975).

These results also suggest that light-mediated conversion of thiarubrine C to thiophene C causes toxic effects on nematodes greater than those resulting from the activity of thiarubrine C or thiophene alone. Thiarubrine C exhibits the properties of a photoactive compound but also has a non-photoactive mode of action, although the activity under light conditions is of a greater magnitude. This finding is similar to the effects of thiarubrine A and thiarubrine C on microorganisms where light promoted the conversion of thiarubrine to the corresponding thiophene, but thiophenes were toxic only when exposed to UV-A light (Constabel and Towers, 1989). In our study, thiarubrine C was very active under dark conditions, as significant mortality was observed in all three nematodes tested. In general, plant-parasitic nematodes seem to be more resistant to nematicides than free-living nematodes. The responses of *M. incognita, P. penetrans,* and *T. dentifera* to thiarubrine C, however, were very different. *Teratorhabditis dentifera* was the most sensitive to thiarubrine C, requiring only 20 ppm thiarubrine C for 8 hours for 100% mortality, while *M. incognita,* at the same thiarubrine concentration, required 72 hours to reach 100% mortality. In comparison, *P. penetrans* required longer exposure times and higher thiarubrine concentrations (72 hours at 50 ppm). Plant-parasitic nematodes, such as *M. incognita* and *P. penetrans,* typically feed on plant hosts; therefore, thiarubrine C would most likely be absorbed through the surface (epicuticle).

Under exposure to UV-A, thiarubrine A was shown to act on membranes (McRae et al., 1985; Hudson et al., 1986). Other studies have shown that the disulfide ring of thiarubrines appears to be of central importance for its activity (Constabel and Towers, 1989). Ring moieties are important for type II photosensitization reactions (McLachlan et al., 1984), which produce long-lived singlet oxy-

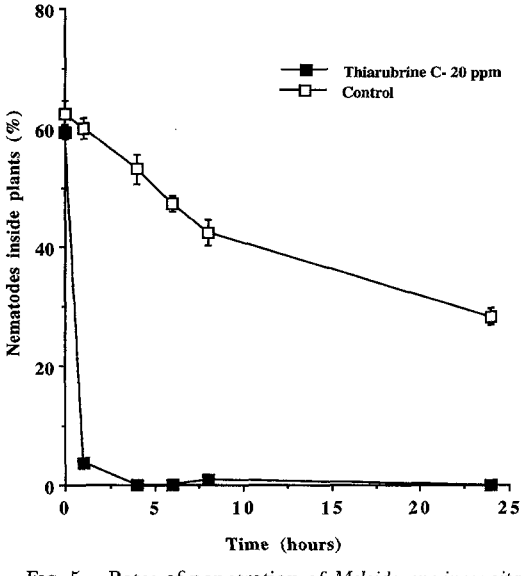


FIG. 5. Rates of penetration of *Meloidogyne incognita* into *Lycopersicon esculentum,* cv. Rutgers, measured after 0, 1, 4, 6, and 8 hours of pre-exposure to 20 ppm thiarubrine C. Plantlet infection was assessed 7 days following infestation. The data points represent the means of two separate experiments (12 replicates per treatment). Bars are standard errors of the means.

TABLE 2. Protective effect of thiarubrine C, applied 0 or 24 hours to soil before transplanting, on infection of tomato plantlets by *Meloidogyne incognita.* Numbers are means ± SE.

Time of soil exposure prior to transplants	Addition of thiarubrine C (50 ppm)	Total nematodes in plantlets
0 hour	–	293 ± 4.3
	+	121 ± 3.9
24 hours	–	300 ± 4.5
	+	32 ± 3.1

gen radicals in the membrane environment (Pooler and Valenzeno, 1979). The release of singlet sulfur during thiarubrine conversion to thiophene in the photoactive process might have contributed to the higher mortality that we observerd under light vs. dark conditions. The fact that thiarubrine C is not totally dependent on light for its toxic effects, however, makes this natural compound more useful as a nematicide.

Gommers and Voor in 't Holt (1976) found that various species of the Asteraceae, including *R. hirta,* reduced population densities of nematodes in both greenhouse and field studies, although no detailed chemical studies to identify the suppressive agents were carried out. Since only members of the Asteraceae produce sulfur-containing polyacetylenes, we tested whether thiarubrine C, a photoactive polyacetylene found in roots of *R. hirta,* is effective against plant-parasitic nematodes in soil. Thiarubrine C retained its activity in soil, suppressing infection of tomato plantlets at concentrations of 50 ppm. Although a 24-hour exposure to thiarubrine C prior to planting greatly reduced infectivity, addition of thiarubrine C at planting was also effective, resulting in a 58.7% reduction in *M. incognita* infection. This work contrasts with reports using terthienyl, a related sulfur-containing thiophene, where the compound was ineffective (Daulton and Curtiss, 1964). This difference likely is due to the fact that thiophene is not active under dark conditions, whereas our results show that thiarubrine C is toxic under both dark and light conditions.

These results show that the ability of root-knot nematode to penetrate and infect the roots of tomato plantlets was greatly reduced in autoclaved soil that previously was treated for 24 hours with 20 ppm thiarubrine C. Although this result is encouraging, thiarubrines are known to be unstable compounds that may be decomposed more quickly in natural conditions. The concentration (20 ppm) used to inhibit plant infection, however, is lower than the amounts required for 100% mortality at longer exposure times in the in vitro study. Thiarubrine C could be acting as both a nematicidal and nematostatic agent, since this concentration typically resulted in 25% mortality of *M. incognita* after 24 hours in in vitro assays. Although the basis for this inhibition of plant infection is not known, thiarubrine C may affect nematode movement or physically damage structures, such as the sensitive stylet muscles (Bunt, 1975), which are essential for penetration and feeding in plant tissue. Further studies will be needed to elucidate the sites of action on nematodes by thiarubrine C.

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