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EVALUATION OF THE ROOT OF DERRIS ELLIPTICA
BENTH. AGAINST NEMATODES

A Thesis Presented

By

BAKTI BIN YUNTON

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University of Massachusetts in partial fulfillment
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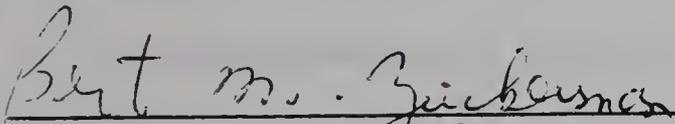
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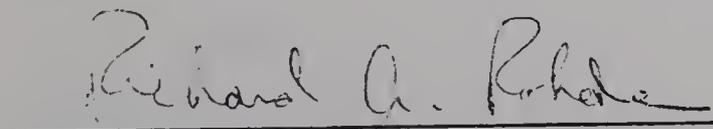
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ABSTRACT

Evaluation of the Root of Derris Elliptica Benth. Against Nematodes

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Derris elliptica Benth. roots contain nematotoxic component(s) extractable with water or polar organic solvents. In immersion tests, aqueous extracts of newly acquired derris roots were nematicidal to Caenorhabditis elegans, Meloidogyne incognita larvae and Aphelenchus avenae at up to 20-fold dilution. The activity of extracts obtained from roots aged in storage was, however, low. Similarly, in greenhouse experiments, soil drenches with aqueous extracts of new roots reduced galling of tomato roots by M. incognita at dilutions up to 20 fold, while extracts from aged roots were ineffective. Extracts at stock and 5-fold dilution levels were toxic to tomato seedlings. Root materials amended to soil at up to 2% by weight basis reduced galling slightly.

Rotenone at up to 500 µg/ml was not lethal to M. incognita larvae and A. avenae. A small number of C. elegans were killed by 500 µg/ml solution after 48 hour exposure.

Acetone and ethanol extracts of the derris roots were highly toxic to C. elegans, and benzene, chloroform and methanol extracts were moderately active. One fractionate from the acetone extract isolated by thin-layer chromatography on Silica Gel G, was toxic to C. elegans and M. incognita. Both species were killed in 24 hours by the fractionate

at 500 ppm (0.25 g root/ml), 750 ppm (0.375 g root/ml) and 1000 ppm (0.5 g/root ml). In a hatching test, the emergence of M. incognita larvae from the eggs incubated in the fractionate solutions were greatly reduced over a 6 day period. These inhibitions persisted after removal of the eggs to water. The isolation of the active principle from the fractionate was not achieved due to the inavailability of fresh roots and degradation of available materials.

Derris root is considered promising against nematodes. For use in a crude form, the material is best applied as extracts for drenching planting sites. Further work with fresh root materials aimed at isolation of the toxic component is warranted.

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CHAPTER I

INTRODUCTION

Some plants are known to be resistant or antagonistic to nematodes. In many cases resistance is dependent on physiological factors of a complex nature. There have been instances however, where resistance relates directly to the inherent presence of nematotoxic substances. In plants such as marigold (Uhlenbroek and Bijloo, 1958, 1959), Eragrostis curvula (Scheffer et al., 1962) and asparagus (Rhode and Jenkins, 1958; Takasugi et al., 1975) the nematotoxic substances have been isolated.

In recent years more and more investigations have emerged around using plants and plant materials for controlling nematodes. Although no nematicide of plant origin has yet been developed for commercial use, this approach has distinct advantages. Isolation of nematicidal components from plants has been successful in many cases because screenings are done selectively on plants of known anthelmintic properties, or on plants which are known to be resistant to nematodes in the field. Once a toxic component is characterized and its actions on nematodes understood, there is the possibility of synthesizing chemical analogues with a similar activity.

An attractive feature of toxic substances isolated from plants is their relative safety to mammals, as seen in the case of the natural insecticides rotenone and pyrethrin. Aside from this, plant materials known to contain nematotoxic substances can be applied in crude form by

incorporation of the plant portions into soil as amendments. This could be a convenient and inexpensive method especially practicable with small gardeners unwilling or unable to invest in expensive equipments. More significantly, the source of the nematicide is renewable by continued replacement planting.

Derris elliptica Benth. of the tribe Dahlbergieae in the Leguminosae is strictly a tropical plant and appears to be native to Southeast Asia and the East Indies (Roark, 1941). In its cultivated form the plant is prostrate, the stem normally trailing and without erect woody side shoots. Chinese gardeners and natives long used the milky juice obtained by beating the roots for spraying crops and poisoning fish (Roark, 1941; Gunther and Turrel, 1942). Extensive research on the plant later established its insecticidal properties, which led to its use as insecticides in the U.S.A. (Roark, 1941).

Rotenone and several related rotenoids isolated from the derris roots (Clark, 1930; LaForge et al., 1933), were found effective against wide groups of insects (Davidson, 1930a, 1930b; Shepard and Campbell, 1932; Roark, 1941). The rotenoids were little investigated however, in light of the much higher insecticidal activity of rotenone, which became the main constituent isolated from the derris root.

The insecticidal activity of derris extracts was usually higher than could be attributed to the rotenone contents alone. Furthermore, rotenone is almost insoluble in water and occurs in the plant cells as discrete water-insoluble particles (Gunther and Turrel, 1942). When derris root is macerated rotenone disperses in water to a very low

concentration. The effectiveness of aqueous extract from the root as used by the Chinese against insects and fish, suggests that other components beside rotenone and the rotenoids may also be toxic. Gunther and Turrel (1942), in a histological and chemical constituent study of the derris root, noted the presence of tannin-like and saponin-like substances. Hemolizing saponins were detected in "quantities that warrant biological and chemical study."

The present study with D. elliptica was encouraged by two main considerations. The root of derris is a recognized poison for fish, mammals and insects alike. Other plants in the family Leguminosae, such as cube, timbo and barbasco also contain rotenone and the rotenoids. None of these plants has been evaluated against nematodes. Rotenone likewise, has been the subject of only a few investigations with nematodes. It is not uncommon that plant materials toxic to insects contain substances active against nematodes. Garlic, for example, which yields an extract toxic to mosquito (Amonkar and Reeves, 1970), also contains components which kill nematodes (Sukul et al., 1974).

For use as a nematicide source material or as a nematode control agent, the plant portion involved should preferably have no other competing use. The plant should also be easily cultivated. Derris more or less fits this criteria. Fish poisoning has been banned in most countries and the only known use of the plant now is as a source of insecticide. In Brunei, derris was used only as fish poison. Currently the plant survives in scattered patches throughout the country, almost in

the wild and with little management. Little difficulty should be encountered in cultivating this plant on commercial scale if required.

Several objectives form the thrust of the current work. Rotenone, although an established insecticide, has received little evaluation as a possible nematocide. The possible toxicity of this substance to nematodes was thereby investigated. Secondly, the investigation was directed at isolating and partially characterizing nematotoxic substances that may be present in the derris root. Finally, it is of interest to evaluate the derris root as a possible nematode control material. Reports on the effectiveness of some plant materials as soil amendments are abundant, and this aspect of application was looked into for derris.

CHAPTER II

LITERATURE REVIEW

Naturally occurring substances toxic to nematodes are reported in many plants. At least eighteen of these have been isolated and identified (Kawazu et al., 1980; Gommers, 1981). Juices and extracts from several plants also exert in vitro nematicidal activity. Isolation work on these will no doubt identify other groups of nematotoxic substances.

Several nematicidal components occur in Compositae. The first isolation in this group was from the marigold Tagetes erecta (Uhlenbroek and Bijloo, 1958). Tagetes species attracted early attention because of their resistance to Meloidogyne (Tyler, 1938) and suppressant effects on Pratylenchus penetrans in the soil (Slotweg, 1956; Oostenbrink et al., 1957). The growing of Tagetes often reduces population of endoparasitic nematodes, and damage to subsequent crops. The active principles in Tagetes are two thiophenes: α -terthienyl (2,2'-5', 2"-terthienyl) and 5-(3-buten-1-ynyl)-2,2'-bithienyl (Uhlenbroek and Bijloo, 1958, 1959). In in vitro assays, these compounds proved toxic to Ditylenchus dipsaci, Anguina tritici, Globodera rostochiensis and P. penetrans. These substances apparently also form a chemical basis for nematode resistance in several other Compositae. Screening work on Compositae which suppressed P. penetrans identified their presence in the genera Berkheya, Didelta, Echinops, Eclipta, Flaveria, Gaillardia and Tagetes (Gommers and van't Holt, 1976).

Synthesis of several analogues followed the isolation of the toxic principles in Tagetes. A number of the derivatives showed excellent nematicidal activity in vitro. The active compounds were all derivatives of 2, 2'-bithienyl (Uhlenbroek and Bijloo, 1960) and of 1, 2-di(2-thienyl)-ethene (Handele, cited in Gommers, 1981). When mixed with soil however, the effects of these substances on nematodes were negligible (Daulton and Curtis, 1963). Similarly, diffusates of Tagetes roots often had low activity against nematodes in the soil (Hesling et al., 1961; Omidvar, 1961, 1962). The suppressant effect of the plant appeared marked only with endoparasites such as Pratylenchus and Meloidogyne species (Hijink and Suatmadji, 1967; Suatmadji, 1968; Hackney and Dickerson, 1975). Nematodes that entered the roots were killed or hampered in their developments (Oostenbrink et al., 1957; Suatmadji, 1968).

Irradiation of α -terthienyl with near ultraviolet light enhanced its nematicidal activity (Gommers, 1972), especially when done under aerobic condition (Gommers et al., 1980). When P. penetrans that had been in Tagetes root were irradiated, they were killed more rapidly than those that had been in α -terthienyl solution (Gommers and Geerligs, 1973). It would appear that in the soil limited photoactivation keeps the α -terthienyl inactive, but within the Tagetes roots the substance is activated by a mechanism other than light.

Other groups of nematicidal substances were subsequently reported in Compositae. Hijink and Suatmadji (1966) recorded the Helenium hybrid "Moerheim Beauty" as resistant to P. penetrans. Later, Gommers (1971)

isolated a benzofuran derivative from the root of the plant. The compound was identified as 2, 3-dihydro-2-hydroxy-3-methylene-6-methyl benzofuran, and was toxic to P. penetrans.

Several acetylenic compounds from Compositae are toxic to nematodes. Two polyacetylenes from benzene extracts of Carthamus tinctorius showed strong in vitro nematocidal activity (Kogiso et al., 1976a, 1976b). The first component, 3-cis, 11-trans-trideca-1, 3, 11-triene-5, 7, 9-triyne killed Aphelenchoides besseyi at 10 ppm within 24 hours. The second component, a 3-trans, 11-trans analogue was lethal to the nematode at 1 ppm. Strong nematocidal activity was also exerted by a red-colored fraction from root extracts of Millieria quinqueflora, Iva xanthiifolia, Ambrosia artemisiifolia, A. trifida, Schkuhria pinnata and Eriophyllum caespitosum (Gommers and voor in't Holt, 1976). The toxic component was tentatively identified as dithio-acetylene.

Two acetylenic compounds were isolated from Cirsium japonicum by Japanese workers (Kawazu et al., 1980). The first component, tridec-1-ene-3,5,7,9,11-pentayne completely inhibited reproduction of Bursaphelenchus lignicolus at a concentration of 16 µg in in vitro assay. The second compounds, 9,10-epoxy-heptadec-16-ene-4,6-dyn-8-01, prevented reproduction at 250 µg. Two compounds, 1-phenylhepta-1,3,5-triyne and 2-phenyl-5-(1'-propynyl)-thiophene from Careopsis lanceolata, and cis-dehydromatricaria ester from Solidago altissima also showed nematocidal activity (Kawazu et al., 1980). Reproduction of B. lignicolus was completely stopped by these substances at a dose of 100 µg.

Phenolics form a chemical basis for nematode resistance in several plants. In cabbage, Acedo and Rohde (1971) reported the accumulation of phenolics in response to P. penetrans infection; hypersensitive reaction to Meloidogyne incognita in tomato similarly correlated with the presence of free phenols (Brueke and Dropkin, 1971). Hung and Rohde (1973) noted higher levels of chlorogenic acid in both healthy and nematode-infected resistant tomato (var. Nemared) as compared to susceptible varieties. Chlorogenic acid was however found to be non-toxic to Meloidogyne larvae and P. penetrans (Pi, 1966), giving rise to the speculation that this substance is converted to the toxic quinone within plant tissues.

In Eragrostis curvula the phenolic, pyrocatechol, is exuded by the root and appears to form the basis for resistance of this plant to root-knot nematodes (Scheffer et al., 1962). Meloidogyne larvae treated with pyrocatechol or root exudate of this plant generally failed to cause infection. In raspberry (Rubus idaeus), polyphenol and tannins were the nematotoxic principles suspected present in the aqueous extracts of the roots and canes (Taylor and Murrant, 1966), but the compounds were not identified.

Phenolics, perhaps more than any other known group of naturally occurring nematicidal substances, show potential for use in field control of nematodes. In greenhouse tests, Taylor and Murrant (1966), incorporated hydroquinone, catechol and resorcinol into the soil and achieved significant reductions in populations of Longidorus and other nematodes. More interestingly, Feldman and Hanks (1971) noted an

increased tolerance to Radopholus similis of grapefruit seedlings that had been root-drenched with phenolics. When vanillic acid was applied the total phenolic contents of infected roots increased to a level comparable with healthy roots. Similarly, a partial resistance against M. javanica was imparted in susceptible tomato plants when cinnamic acid and catechol were applied as soil drench, foliar spray or root-dip treatment (Sitaramaiah and Pathak, 1979). These results suggest a possible systemic activity of some phenolics, which may enhance the usefulness of these compounds as nematicides.

Incorporation of various organic materials in soil has been found to reduce nematode populations in many cases. The extensive literature on these was reviewed by Singh and Sitaramaiah (1970, 1973), and will only be considered here as they pertain to phenolics and the present work. The suppression of nematode activity in soil amended with organic materials is attributed to one or more factors:

- (1) increase in number and activity of soil organisms antagonistic (predatory, parasitic, competitive) to nematodes,
- (2) production of nematotoxic metabolites by microbes active during the decomposition processes,
- (3) the organic material may be directly toxic to the nematodes or in some manner adversely affect nematode physiology,
- (4) the decomposition products of the amendments may be toxic to the nematodes,
- (5) alteration in soil temperature, pH, oxygen and nitrogen status to that unfavorable for nematode activity, and

(6) physical and chemical conditions of the soil may be changed, contributing to increased tolerance of host plant to nematodes. Improved soil conditions may enhance root development and nutrient uptake and this would mask the effect of nematode injury. Unfavorable alterations in osmotic relations in the soil solutes may also occur, which can induce phenomena like ovoviparity, to result in reduction in the numbers of infective larvae.

Amendment with mahua (Madhuca indica), castor (Ricinus communis), mustard (Brassica campestris), neem/margosa (Azadirachta indica) and groundnut (Arachis hypogaeae) oil cakes reduced nematode damage to okra and tomato in field trials in India (Singh and Sitaramaiah, 1970, 1973). Neem contains the phenolics nimbidin and thionimone, both of which were toxic to nematodes and inhibitory to larval hatch (Khan et al., 1974). Similarly, water soluble fractions of oil cakes of mahua, groundnut, castor and neem were nematocidal and inhibited larval emergence. In Nigeria, neem aqueous extracts were used as soil drench for effective reduction of P. brachyurus around maize roots (Egunjobi and Larinde, 1975; Egunjobi and Afolami, 1976). Alam et al. (1979) found high concentrations of phenolics in these oil cakes and in mustards. Ten phenolic compounds tested by these workers all showed nematocidal activity, particularly hydroquinone, p-cresol, catechol, pyrogallol and gallic acid. The effectiveness of sawdust as soil amendment may partially be due to its phenolic contents. Singh and Sitaramaiah (1973) reported an increased level of phenolics in soil amended with sawdust.

It would appear that amendments high in phenolic contents achieve reduction in nematode damage by direct toxicity to the nematodes (Alam et al., 1979) or by favorable effect on the host physiology, such as by imparting partial resistance in the host plants (Feldman and Hanks, 1971; Sitaramaiah and Pathak, 1979).

Several species of Asparagus are antagonistic to nematodes. A. officinalis var. atilis L. was found to be highly resistant to Trichodorus christiei. A nematicidal glycoside with systemic activity was isolated from the roots (Rhode and Jenkins, 1958). The nematicidal action of the substance was probably related to its inhibition of nematode acetylcholinesterase (Rohde, 1960).

Swarup and Sharma (1967) reported nematotoxicity in aqueous extracts of A. racemosus roots. The extracts inhibited hatching of M. javanica and M. arenaria eggs in vitro. Isolation and identification of the toxic component were, however, not attempted.

Using a modification of Rhode's isolation procedure for glycoside, Takasugi et al. (1975) were able to isolate asparagusic acid (1,2-dithiolane-4-carboxylic acid) as an active nematicidal principle in asparagus. The acid inhibited larval emergence from cysts of Heterodera rostochiensis and H. glycines even in the presence of a hatching stimulant, and was lethal to larvae of H. rostochiensis and M. hapla and larvae and adults of P. penetrans and Paratylenchus curvittatus at 50 ppm concentration.

Nematicidal alkaloids are recorded in a few plants. Physostigmine, an alkaloid from Calabar bean (Physostigma venenosum), inhibits

the motility of Ditylenchus dipsaci in vitro and exerts a systemic nematicidal activity (Bijloo, 1965). This systemic activity is also characteristic of several analogues, notably 3,5-dimethyl-4-dimethylaminomethylphenyl N-methylcarbamate, which is more active than physostigmine (Welle, 1964). The systemic nematicidal activity of these substances has been ascribed to their inhibition of the nematode cholinesterase.

Solanum tuberosum contains the steroid-glycoalkaloid α -charconine, which is toxic to Panagrellus redivivus. A study with a buffered solution of the substance showed that its nematicidal activity increased with increasing pH, thereby establishing the free base as the nematicidal form (Allen and Feldmesser, 1971). Nematicidal activity of α -tomatine from tomato is similarly pH-regulated (Allen and Feldmesser, 1970).

Crotalaria species are known to suppress Meloidogyne. Monocrotaline, a pyrrolizidine ester compound which was isolated from C. spectabilis inhibited the mobility of M. incognita larvae (Fassuliotus and Skucas, 1969). The substance was not considered directly related to resistance however, since some other plants which contain this compound, such as Cytisus and Echium species are susceptible to Meloidogyne.

Three alkaloids identified as sanguinarine, cheletyrine and bocconine isolated from the roots of Bocconia cordata showed strong nematicidal activity against Rhabditis and Panagrolaimus species (Onda et al., 1956, 1970).

Japanese workers isolated from sweet daphne (Daphne odora), two compounds which were toxic to A. besseyi. The compounds odoracin and odoratrine were both lethal to the nematode at 1 ppm (Kogiso et al., 1976c; Munakata, 1979). Their diacetate and isopropylidene derivatives, although chemically similar, were not nematocidal (Munakata, 1979). This led Gommers (1981) to believe that the nematocidal activity in these compounds was dependent on OH-groups.

In cotton, resistance to M. incognita is afforded by terpenoid aldehydes (Veech, 1979). The compounds accumulate rapidly in the root in response to nematode invasion, and are lethal to young larvae.

Mustard, Brassica nigra, contains the oil allyl isothiocyanate which apparently is toxic to nematodes. Allyl isothiocyanate incorporated into soil in a peat carrier, increased the yield of potato markedly (Ellenby, 1945, 1951). The oil reduced hatching of H. rostochiensis cysts in vitro; in the field the number of cysts around potato roots were reduced and plant growth improved (Ellenby, 1951).

Various constituents of peach tree roots are believed to contribute to the "peach decline" problem in certain areas (Israel et al., 1973). The roots contain amygdalin, which following root injury, is hydrolyzed to release HCN (prussic acid) and benzaldehyde to the soil. Benzaldehyde, KCN and peach root bark applied to the root region, inhibited the growth of young peach cuttings. These materials as well as amygdalin were also toxic to soil microbes and nematodes. This study was not extended however, to determine if these substances are toxic to other plants, and if they are nematocidal at a concentration that is not phytotoxic.

Nematicidal substances that have been discovered in plants belong to the groups alkaloids, glycosides, phenolics, sesquiterpenes, diterpenes, polyacetylenes, isothiocyanates, thiophenes and other sulphur containing compounds. Many other plants besides, contain nematotoxic components that are not yet identified. Amongst these, toxicity has been reported in extracts of some crop plants.

Sukul et al. (1974) observed strong nematicidal activity of extracts of ginger, garlic and chili pepper in an in vitro assay and in a root drench pot experiment. While edible crop produce may not be of direct application in field control of nematodes, the isolation and identification of the toxic component could enable synthesis of analogues which may show similar toxic activity. The nematicidal properties of leaf extracts of bean (Phaseolus vulgaris), tobacco (Nicotiana tabacum), corn (Zea mays), tomato (Lycopersicon esculentum) and rhododendron (Rhododendron catawbiense) noted by Miller et al. (1973) may have interesting applications. These inedible portions of the crop plants can be incorporated into the soil for nematode control. Homogenized leaves of bean, tobacco and tomato amended to the soil were found by these investigators to significantly reduce populations of Tylenchorhynchus dubius within a few days. Evaluation, of course, must be done to determine the influence of these materials on the build-up of soil pathogens.

The common vegetable oils used in cooking have adverse effects on nematodes. Miller (1979) mixed corn oil, cottonseed oil, linseed oil, olive oil and safflower oil with soil, and achieved protection of tomato

plants against P. penetrans at a rate as low as 1 ml oil/kg soil. Soybean at 1 ml/kg soil reduced T. dubius populations considerably, and was not phytotoxic. Quite strikingly, corn oil afforded a systemic type of protection. The oil at 0.25% in water, used as a root-dip treatment for tomato, reduced penetration of the roots by P. penetrans. These oils may be feasible for use in a limited scale application, such as in home gardens or lawns.

Pangola grass (Digitaria decumbens) is known to be resistant to root-knot nematodes. Extracts and exudates of pangola grass roots were toxic to larvae and inhibited hatching of M. incognita (Haroon and Smart, 1980). When interplanted with tomato, the grass reduced galling in the tomato roots, and suppressed the nematode population in the soil more effectively than fallow.

The leaves of Polygonum hydropiper contain substances antagonistic to nematodes. The active compounds are also exuded in root diffusates (Sukul, 1970). Growing P. hydropiper together with wheat inhibited infestation of the crop plant by Anguina tritici.

Not surprisingly, many anthelmintic plant materials exert nematocidal activity. Fruit juice of Duranta repens at 0.4% concentration was toxic to Hoplolaimus, Tylenchorhynchus, Ditylenchus, Meloidogyne and saprozoic nematode species (Husain and Saxena, 1969). Fruit juices of Solanum xanthocarpum and Argemone maxicana, and seed extracts of Cucurbit pepo and C. maxima exerted similar activity at slightly higher concentrations.

Corm powder of Typhonium trilobatum, a commonly used concoction for filariasis treatment in India, was effective against M. incognita (Mukhopadhyaya et al., 1980). The powder, incorporated at 0.3 g and 0.6 g per 1 kg of pot soil, reduced galling in eggplant better than D-D (1,3-dichloropropene-1,2-dichloropropane mixture). In addition, the residual effect of one application extended to a second crop, with plant growth similarly improved and nematode damage greatly reduced.

Husain and Masood (1975) evaluated extracts from other plants of known anthelmintic properties. Leaf extracts of blumea, margosa, wormseed, tagetes and jasmine; seed extracts of white lupine, lima bean, snake-gourd, cabbage, black cumin, pumpkin, embelia and papaya; ergot extract of pearl millet; and flower extracts of Cuscuta and worm-wood were all toxic to Tylenchus filiformis, Helicotylenchus indicus and Tylenchorhynchus brassicae. Margosa, blumea and worm-seed leaf extracts showed higher toxicity than tagetes.

Wild plants of no reported economic use provide another source of nematicidal substances. Chatterjee and Sukul (1980), found nematicidal activity in petroleum ether extracts of dried leaves of Tragia involucrata (Euphorbiaceae), Anthocephalus kadamba (Rubiaceae) and chloroform extract of Peristrophe bicalyculata (Acanthaceae). Extracts from A. kadamba were the most potent, and all three were more toxic than aldrin, which is a poor nematicide.

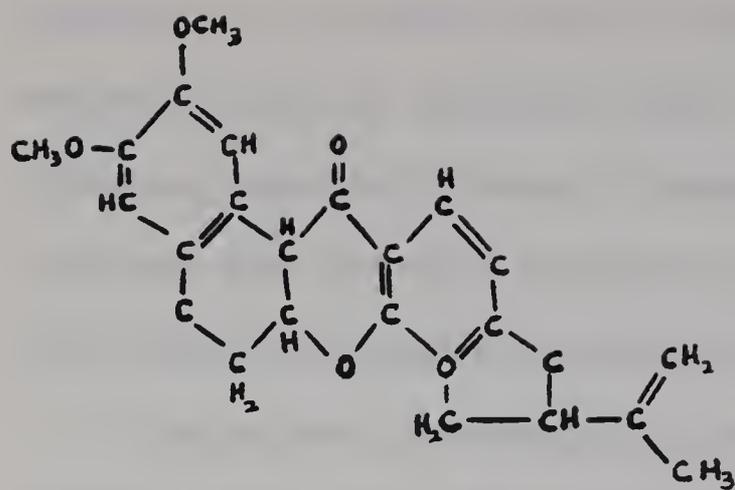
Derris elliptica Benth. contains substances toxic to insects, fish and mammals, but has not been evaluated against nematodes. Rotenone, the main insecticidal constituent, was described by LaForge et al.

(1933). Five other crystalline substances, referred to as "rotenoids" (Roark, 1940) in view of their structural resemblance to rotenone, are also obtained in the extraction of derris root. Deguelin and toxicarol were isolated and described by Clark (1930); sumatrol, elliptone and malaccol respectively by Cahn and Boam (1935), Harper (1939), and Meyer and Coolhas (1939). Tephrosin, also a rotenoid, was sometimes isolated but appeared to be a breakdown product of deguelin (Clark, 1930). All of these substances are chemically related (see Fig. 1), but they differ greatly in their toxicity to insects.

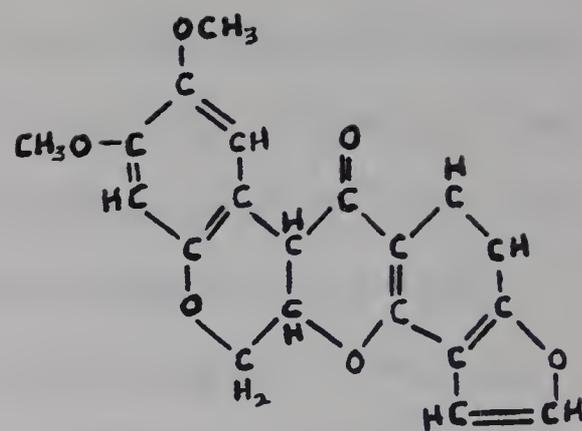
Rotenone is a highly potent stomach and contact insecticide (Davidson, 1930a; Shepard and Campbell, 1932). The M.L.D. (Median lethal dose) on silkworm was reported as 0.003 mg/g, thirty times as toxic as lead acetate. Wide groups of insects are affected by rotenone but the effect seems to be specific. Some insects feed readily on the roots of rotenone-containing plants (Jones, 1938); others, such as Melanoplus femur-rubrum are resistant to the substance (Richardson and Haas, 1932).

Rotenone is highly sensitive to light and air. Derris dusted or sprayed to plant deteriorated relatively fast under sunlight, and more slowly under shade. Residues of latex emulsion, derris powders and water suspensions of derris powders applied to bean plants lost their activity within ten days (Pagan and Morris, 1953). Todd (1938) reported that a 0.75% rotenone dust retained activity longer than two weeks in the shade, but was not toxic after one week under direct sunlight. In solution, rotenone turns from colorless to yellow as it decomposes on

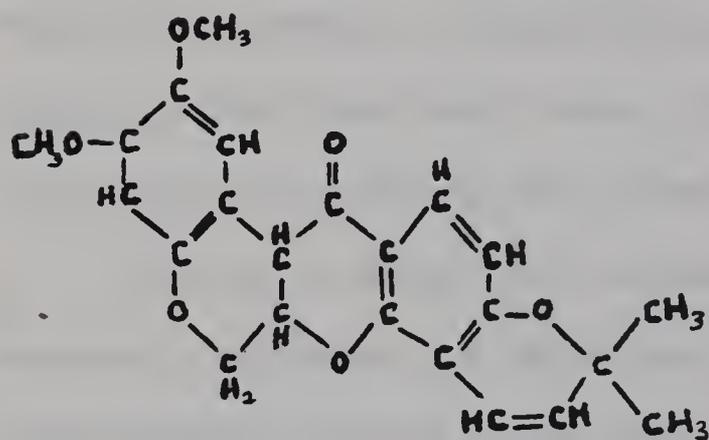
Fig. 1. Rotenone and related rotenoids.



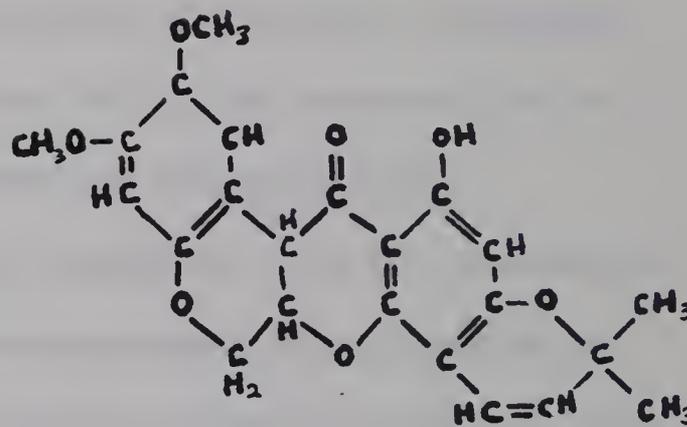
Rotenone



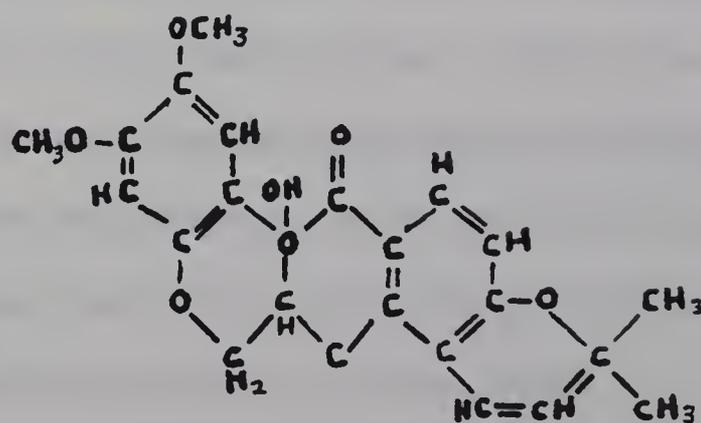
Elliptone



Deguelin



Toxicarol



Tephrosin

standing. This occurs quickly in some solvents, but only slowly in acetone or alcohol (Gunther, 1943). A change from the basic rotenone structure invariably leads to reduction in toxicity. Of the at least 20 compounds that rotenone decomposes to on exposure to light, only 6 $\alpha\beta$, 12 $\alpha\beta$ -rotenolone retains significant toxicity (Cheng et al., 1972).

The actions of rotenone on insects were reported in several studies. The main effect appears to be an interference with the respiratory processes (Tischler, 1935). Damage to motor nerves and muscles occurs infrequently. Tissue disintegration was noted in Thermobia domestica which developed latent injury following recovery from sub-lethal doses of rotenone dust (Sweetman and Gyrisko, 1944).

There is evidence that rotenone interferes with the respiratory process in nematodes. A study with a mitochondrial preparation of Caenorhabditis elegans showed that rotenone inhibits state 3 respiration, in common with the mammalian respiratory inhibitors cyanide, azide and Actimycin A (Murfitt et al., 1976). The effect of rotenone on live nematodes has not been widely studied. Rich et al. (1977) did not find this substance nematicidal to Pratylenchus scribneri, but it caused a slight inhibition of movement. This test was conducted with one concentration (50 $\mu\text{g/ml}$) and over a relatively short exposure period. The effect of rotenone on insects is often slow.

Several of the rotenoids in derris root possess insecticidal activity. Deguelin and tephrosin were as toxic to silkworm as acid lead arsenate, and toxicarol slightly less (Shepard and Campbell, 1932). None of these substances however approached the activity of rotenone.

In the derris root, rotenone may be present to about 4-9%, and it constitutes up to 40% of the derris resin. In view of this, rotenone became the choice component for isolation as an insecticide. Few works on the rotenoids followed the initial investigations.

Gunther and Turrel (1942) reported the presence of tannin-like and saponin-like substances in the derris root. Hemolyzing saponins were found in substantial amounts. Since rotenone occurs in discrete water-insoluble particles in the derris cells, and only disperses in water to a very low concentration when the root is macerated, they suspected that substances such as these may be partially responsible for the effectiveness of the derris root extract as used for fish poison by natives.

The solubility of rotenone in water is less than 1 ppm. Up to 31% of the active material may however be removed in suspension by water extraction if the root has been finely ground (Goodhue and Haller, 1939). A more common method of extraction is by using organic solvents. Rotenone dissolves in chloroform at up to 47.2 gram per 100 ml at 20°C (Jones and Smith, 1930). Benzene dissolves 8.0 gram rotenone per 100 ml, acetone 6.6 gram. Alcohols and ether are generally poor solvents for rotenone. In contrast to the rotenoids, rotenone forms crystalline solvate with benzene, carbon tetrachloride, acetic acid and chloroform. This property facilitates its isolation from the derris resin.

Acetone, chloroform, benzene or ether is normally used in the extraction of derris root. The procedure described by Jones and Graham

(1938) has now been adopted as standard. This procedure calls for extraction of finely ground derris root by "Multiple Extraction Method" or by "Aliquot Method." In the Multiple Extraction Method, the derris sample is refluxed repeatedly with the solvent at room temperature or in a steam bath. In the Aliquot Method the sample is shaken in a measured volume of solvent at room temperature for 2-24 hours. The extract obtained by either method is filtered, and evaporated to dryness after most of the solvent has been removed by distillation. Rotenone may be recovered by crystallization with carbon tetrachloride, and is further purified with ethanol.

In general chloroform, acetone and methyl alcohol give satisfactory extractions of rotenone (Jones and Sullivan, 1938; Jones and Graham, 1939). Chloroform extracts yield relatively small amounts of other derris constituents, and for the purpose of rotenone extraction alone it is generally preferred. For total extractives, acetone or methyl alcohol is more efficient (Jones and Sullivan, 1938).

Thin-layer chromatography has been employed for the separation of rotenone and rotenoid mixtures. Delfel (1966) found Silica Gel G developed with chloroform-acetic acid mixture (199:1) useful for separation of some of the compounds. The spots were identified by their characteristic colors which appeared within minutes of spraying with hydriodic acid or after heating following the spray. For specific separation of rotenone, elliptone and tephrosin, Silica Gel G slurried with 12.5% silver nitrate solution was found useful. To locate the positions of the compounds, the plates were either sprayed with

concentrated nitric acid and heated at 120°C for 15 minutes, or they were left exposed to air and subdued light for several hours.

A slight modification to the same procedure was later made by Delfel and Tallent (1969) for a more effective separation. The slurry for coating the plate was prepared by mixing 1 part silica gel in 2 parts (w/v) silver nitrate solution (24% in water). By use of a special spreader a two thickness coating was made on the same plate. Samples for separation were run on lanes on the thinly coated portion of the plate, and the solvent front was allowed to run to the thicker portion. Since the thicker coating could absorb more solvent, this allowed for an extended development period to achieve the effect of a longer development distance. The developing solvent system used was a 196:3:1 mixture of chloroform, acetone and acetic acid. Exposure of the developed plates to nitric acid vapor, followed by ammonia vapor caused the substances to yield dark colored spots. Rotenone, tephrosin, deguelin and elliptone separated well from each other, but deguelin was not separated from toxicarol or sumatrol. Separation of the compounds in the derris extract may not be as satisfactory however, due to the presence of other constituents.

CHAPTER III

MATERIALS AND METHODS

Nematode Source

Aphelenchus avenae. Culture of A. avenae was initially started from a single adult female isolated from an agricultural soil taken at the University of Massachusetts Amherst campus. The nematode was surface-sterilized by immersion in a 0.1% Malachite green solution for 15 minutes followed by rinsing in sterile distilled water. It was then transferred to petri dish culture of Rhizoctonia solani on Potato Dextrose Agar, and incubated at 23°C.

When specimens of the nematode were required, individuals were obtained by washing the culture with a small volume of sterile distilled water. The nematode suspensions were placed in a Baermann funnel, and the nematodes collected in 6 hours. They were screened on a 325 mesh sieve to remove out larvae. The individuals retained on the sieve were used in the experiments.

Caenorhabditis elegans. Culture of C. elegans was initiated from pure stocks maintained at Dr. Bert M. Zuckerman's laboratory at the University of Massachusetts. The nematodes were cultured axenically in a growth medium prepared with 4 g yeast extract, 3 g soytone and 10 ml fresh live extract in 90 ml sterile water (Sayre et al., 1963).

The nematodes used in the various bioassays were individuals approximately 4 days old, obtained from age-synchronized cultures. The

culturing technique for this followed the procedure of Zuckerman et al. (1971). Egg masses were removed from stock cultures, rinsed three times in sterile distilled water and transferred to nutrient growth medium in culture vials. The vials were incubated at 22°C. At 5 days, the nematodes were freed from the nutrient solution by 4-hour processing in the Baermann funnel. The collected nematode specimens were centrifuged for 5 minutes at 5000 rpm to obtain a concentrated suspension.

Meloidogyne incognita. The root knot nematode used in this work was M. incognita race I obtained from Dr. Sasser at North Carolina University, Raleigh. The nematode was maintained in the greenhouse on potted tomato (Lycopersicon esculentum), var. Rutgers.

Tomato seeds were surface-sterilized for 5 minutes in a 10% Chlorox solution, rinsed in sterile water and germinated on moist filter paper in a petri dish. Germinated seeds were transferred to autoclaved soil in 8-inch pots. At 1 month old, the seedlings were inoculated with egg masses placed directly on the roots. New cultures were started at intervals.

The tomato plants were fertilized weekly with 20-20-20 "HYPONEX" soluble fertilizer. The temperature in the greenhouse ranged from 20°C-30°C in the day; daylight extended from 10-14 hours in the course of the work.

Larvae for use in the experiments were obtained from 6-10 week old cultures. Egg masses were removed from the tomato roots by teasing and picking with needles. The egg masses were immersed in a 0.1% Cetavlon

solution for 5 minutes, rinsed in sterile water and further soaked in 0.5% Hibitane diacetate solution (Peacock, 1959). Following a series of final rinsings, the egg masses were placed in sterile distilled water in watch glasses and incubated in the dark at 23°C. Hatched larvae were removed daily with pipettes. Larvae used in the experiments were freshly hatched within 24-48 hours.

Extractions of Derris Roots

Roots of Derris elliptica Benth. were obtained from 2-year old plants grown in Brunei. The roots were air-dried under the shade before dispatch to Massachusetts. Due to time in delivery, the roots were at least 3 weeks after harvest when first used. They were stored in dark plastic bags at 5°C for up to 6 months before being discarded. The roots were taken from plants in the same field, but were received in 2 separate batches at 5 months interval.

Extraction with organic solvents. Acetone, benzene, chloroform, ethanol and methanol were used in preliminary extraction works. The solvents were freshly glass-distilled.

Roots were cut into pieces of about 5 mm length and macerated in 10 times the volume of solvent (w/v) in a Waring Laboratory blender. The blender was run at low speed for 2 minutes followed by 1 minute at high speed. The ground root materials were placed in Erlenmyer flasks and the flasks wrapped with aluminum foil. Flasks were shaken mechanically for 17 hours. Following this the derris marcs were squeezed in a

4-layer cheesecloth to extract as much juice as possible. The marcs were re-extracted with fresh solvent.

The extracts from the two series of extractions were combined and filtered through a filter paper (No. 41H "Ashless") by vacuum suction. The extract was then reduced to 1/40 of the volumes of solvent originally used in extraction in a vacuum evaporator. One ml of the final extract represented a concentrated extract from 2 g root. The solvents were evaporated in minimal heat: ethanol, benzene and methanol at 45°C; acetone and chloroform at 35°C. Where complete removal of the solvent was required, drying was done by normal evaporation under the fume hood.

Extraction with water. The same general procedure was followed for extraction of roots with water. However, the roots were ground in the blender with 3 volumes of glass-distilled water per g root material, and without further shaking. After it was extracted from the derris marcs, the extract was centrifuged at 10,000 rpm for 10 minutes to remove solid particles.

The extract obtained above was arbitrarily designed as "stock" (S), and further dilutions as required were made with sterile distilled water. To prevent bacterial contamination, streptomycin sulphate at 1000 ppm was added to the extracts. The extracts were frozen at -10°C when not in use.

Laboratory Assays of Derris Extracts

Water extract. Stock (S) derris aqueous extract was obtained by extracting 10 g root newly acquired from Brunei. Five-fold, 10-fold,

20-fold and 40-fold dilutions were made with sterile distilled water. These extract preparations were designated as S, 1/5 S, 1/10 S, 1/20 S and 1/40 S respectively. Streptomycin sulphate was added separately to the extract at 1000 ppm. Due to the sensitivity of C. elegans to this substance, extracts for bioassay against C. elegans were not treated.

The bioassay was done by "Immersion Test." A 10 μ l suspension of nematodes (40-50 individuals) was introduced with a micropipette into a 1 ml Bureau of Plant Industry (BPI) watch glass, and 0.5 ml of the test extract was pipetted into the watch glass. This was replicated 5 times for each level of extract strength with each test nematode species. The same number of replications with sterile distilled water containing 1000 ppm streptomycin sulphate (or untreated sterile distilled water in bioassays with C. elegans) served as controls. The watch glasses were arranged inside petri dishes on moist filter paper. The petri dishes were stacked one above the other inside an incubator set at 23°C and without lighting.

The extracts were tested against adults of A. avenae, young adults (4 days old) of C. elegans and second stage larvae of M. incognita. The nematodes were examined under a stereoscope at 6, 24 and 48 hours of incubation. Counts for toxicity were made as percentage of nematodes that were killed or completely immobilized. The nematodes were considered dead if they assumed a characteristic rigid posture and did not respond to gentle probing with a needle.

As in the course of the present work, it was noticed that aqueous extracts showed variable activity, the tests were repeated using

extracts obtained from roots obtained from roots that had been in storage for 2 months.

Organic solvent extracts. Two grams of derris roots were separately extracted with 100% ethanol, acetone, benzene, chloroform or methanol by the described procedures. The extracts were completely freed of solvent and the remaining residues redissolved with minimal amounts of 100-120 μ l) of ethanol, except for residues from benzene and chloroform extractions, which were taken up in acetone. Each was then diluted to 1% of the solvent or less with 8 ml of sterile distilled water. The resulting suspensions with precipitated materials were shaken overnight and then centrifuged at 5000 rpm for 10 minutes. The clear supernatants were removed for bioassays.

The same bioassay procedure as described for water extracts was used. Test nematodes were again the same three species. The extracts were tested only at one (stock) strength, with 4 replicates for each nematode species. Nematodes in 1% acetone were the controls.

Bioassays of Rotenone

Commercial rotenone (Sigma Chemical Company) was dissolved in 1% acetone at 500 μ g/ml, 250 μ g/ml, 100 μ g/ml and 50 μ g/ml. These were bioassayed against A. avenae, M. incognita and C. elegans by the Immersion Test procedure. One percent acetone was the control. Each treatment was replicated 5 times.

Thin-Layer Chromatography of Derris Extracts

Standard chromatography procedures (Randerath, 1965) were followed in all chromatography work. Solvents were glass-distilled at least once. Development tanks were lined along the inside walls with filter paper, and developing solvents were maintained to submerge the plate by 2 mm. Chromatography was carried out at room temperature in dim light.

Plates were coated with 0.25 mm Silica Gel G or Silica Gel HF₂₅₄ (fluorescent). Silica Gel G was slurried in water at 30 g/60 ml, and Silica Gel HF₂₅₄ at 30 g/75 ml. The plates were activated for 90 minutes at 120°C in the oven and used immediately on cooling in a desiccator.

In preliminary works, acetone, chloroform and ethanolic extracts of derris were chromatographed beside each other to compare spots. The position of rotenone was identified by running the purified rotenone (Sigma Chemical Company) standard. Fluorescent spots on Silica Gel HF₂₅₄ chromatograms were identified under UV light (wavelength 3660 Å). To visualize spots, the developed chromatograms were sprayed with potassium permanganate or iodine solution (Randerath, 1965), or hydriodic acid, a reagent useful for rotenone and rotenoids (Delfel, 1966). Potassium permanganate was prepared by dissolving 0.5 g of the substance in 15 ml concentrated sulfuric acid; iodine solution by dissolving 0.5 g iodine in 100 ml chloroform. Hydriodic acid was a mixture of 1 volume of 5N potassium iodide solution with 30 volumes of 45% phosphoric acid. Following treatment with hydriodic acid the plates were heated for 20 minutes at 120°C. Chromatography for collection of extract fractionates

for bioassays were done on Silica Gel G, without reagent spray or heating.

Acetone extracts. Similar chromatograms were yielded by acetone, chloroform and ethanolic extracts of derris roots. Acetone extract was chosen for use in subsequent chromatographic studies.

Extracts from 10 g of root were concentrated to 2.5 ml. These were chromatographed on Silica Gel G after initial identification of spot locations on Silica Gel G and Silica Gel HF₂₅₄ as described. A 50 µl extract was spotted with a micropipette on each plate at 1 cm intervals and 2 cm above the bottom edge of the plate. The plates were developed in 9:2 benzene-acetone for a distance of 15 cm (development time 35 minutes).

Spots were scraped with the gels from positions identified earlier. Portions of the chromatograms which did not yield spots were scraped off in sections approximately 1 cm wide. A 1 cm section was removed from below the base line. Eluant from this was to serve as control in bioassays of fractionates for toxicity.

Scraped gels were separately eluted twice in 3 volumes of acetone. The acetone was filtered and evaporated to 1 ml at 35°C. This was dried under the fume hood to leave behind the fractionate residue. The residue was weighed and redissolved in a minimal amount of acetone. Sterile distilled water was added to dilute to 2% acetone. With all fractionates, the final concentration of the fractionate in the solution was not less than 2000 µg/ml. Each fractionate solution was filtered through a 0.22 µm Millipore filter and assayed for toxicity.

Water extracts. Similar procedures as for acetone extracts were followed for chromatography of aqueous extracts of derris. The plates were developed for a 10 cm distance in 9:2 benzene-acetone. Scraped gels were eluted twice in 3 volumes of ethanol. Fractionate residues were redissolved in minimal amounts of ethanol and the solution diluted to 2% ethanol with sterile distilled water. The fractionates were assayed against C. elegans and M. incognita.

Bioassay results with fractionates obtained from small amounts of extracts (2 g root equivalent each time) were found negative in a few series of isolations and tests. Chromatographic work with aqueous extract was thereby discontinued. Occasional tests of water extracts also showed a low toxicity. This activity was practically absent in water extracts of roots that had been previously extracted several times with acetone.

Bioassays of Fractionates for Toxicity

Bioassay of fractionates from acetone extracts was by the Immersion Test. Twenty to forty young adults of C. elegans and larvae of M. incognita were placed together in the same watch glass in 100 μ l of sterile distilled water. An equal volume (100 μ l) of the test fractionate solution was added. Controls were provided by eluant of Silica Gel G in 1% acetone. Each treatment was replicated 3 times and the test continued for 72 hours.

Activity of Toxic Fractionate 1

Toxic fractionate 1, which was identified in the above test, was further evaluated separately against C. elegans and M. incognita. The fractionate was tested at 1000 ppm, 750 ppm, 500 ppm, 250 ppm and 100 ppm. Since 2000 µg of the fractionate was obtained in extraction from 1 g root, these approximately represented an equivalent of 0.5, 0.375, 0.25, 0.125 and 0.05 g root per ml solution respectively. A 1% acetone solution was run as controls. Twenty to thirty nematodes were placed by needle in 0.1 ml of the test solution in a BPI watch glass. The treatments were replicated 5 times. The test with C. elegans was conducted for a 48-hour period and with M. incognita for 72 hours. Nematode activities were observed occasionally in the first few hours, and at 24, 48 and 72 hours.

Effects of Toxic Fractionate 1 on Hatching

The toxic fractionate was evaluated for its effects on hatching of M. incognita eggs. A 0.2 ml solution of the fractionate in 1% acetone was placed in a watch glass. Three surface-sterilized egg masses were placed inside each watch glass. The fractionate was tested at 1000 ppm (0.5 g root/ml), 750 ppm (0.375 g root/ml) and 500 ppm (0.25 g root/ml). Acetone 1% served as the control treatment. Each treatment was replicated 4 times. The 16 watch glasses were placed inside petri dishes and incubated in the dark at 25°C.

The number of larvae that emerged were counted daily over a 6-day period. After the last count, the incubation solutions were removed and replaced with sterile distilled water. The eggs were allowed to hatch in the water for an additional 4 days. The water was then replaced with a 10% Chlorox solution. The remaining unhatched eggs, which were released on dissolution of the egg matrices in 1-2 hours, were counted. The percentage of hatching throughout the experiments were calculated based on the total number of eggs in the egg masses.

Attempts at Characterizing Toxic Fractionate 1

Toxic activity was found absent in similar fractionate collected from other samples of the same root batch in a subsequent isolation. Since new roots were not available, isolation of the active component of the fractionate for characterization was attempted using two separate samples.

Sample 1 was obtained by air-drying the available toxic fractionate solution (from the first toxic isolation). The fractionate solution had been in storage at 5°C for 5 days but was still active. The fractionate residue left after removal of the water was redissolved in acetone at 10,000 µg/ml.

Sample 2 was taken from fractionate material from the non-toxic isolation. The fractionate had not been dissolved in water. The material was similarly dissolved in acetone at 10,000 µg/ml.

Both samples were chromatographed on Silica Gel G and the plate developed in 9:2 benzene-acetone. The spots were identified after

spraying with hydriodic acid and heating at 120°C for 10 minutes. The samples were further chromatographed on Silica Gel G developed in chloroform:acetic acid (199:1) for a 10 cm development distance. Rotenone was run for comparison. The procedure was repeated for chromatography on Silica Gel HF₂₅₄. Spot positions were identified by hydriodic acid and iodine solution sprays, followed by heating. Chromatograms on Silica Gel HF₂₅₄ were examined under UV light (wavelength 3660Å).

After the preliminary identification of spots positions above, further chromatography was done on Silica Gel G without the subsequent chemical spray or heating. Separated components and the base spotting sections were removed separately by scraping the gel. The gels were eluted with acetone, and the components recovered after evaporation of the solvent. The various components were weighed and each redissolved in 1% acetone at 500 ppm concentration. They were assayed against M. incognita larvae and C. elegans young adults.

Greenhouse Assays of Derris Root Materials

Soil type and preparations for the various greenhouse pot experiments were similar in all tests, except where otherwise specified. The soil used was autoclaved sandy loam (71% coarse sand, 16% fine sand, 8.6% silt and 4.4% clay) with an initial pH of 4.7. Lime was incorporated at 5 g/kg soil.

Tomato var. Rutgers was chosen as the test plant. Seeds were surface-sterilized for 5 minutes in a 10% Chlorox solution, followed by

rinsing in several changes of sterile distilled water. The seeds were pregerminated in petri dishes, then transferred to soil trays. Seedlings were generally 2-4 weeks old when transplanted to pots.

Nutrients in the form of HYPONEX 20-20-20 soluble fertilizer were supplied to the plants from the first week. Each week plants in the pots received 40 ml of the solution, or 50 ml of half-strength Hoagland's solution (Hoagland and Arnon, 1950) which was applied at every two weeks.

The temperature in the greenhouse ranged from 20-30°C. Daylength extended from 10-14 hours (winter to spring months). Artificial lighting was not used to supplement daylight.

Drench experiment 1. Fifty 200 ml styrofoam cups were filled with autoclaved soil. The soil was inoculated with 500 freshly hatched larvae of M. incognita per cup by pipetting the nematodes in a 3 ml suspension into holes made 3 cm deep. The soil was kept slightly moist. Two days later 10 cups were each drenched with 30 ml of stock (S), 1/5 S, 1/10 S or 1/20 S extract of derris root, or sterile distilled water. The extracts were obtained from roots newly received from Brunei. When the soil had dried slightly, a 2-week old tomato seedling was transplanted into each cup. Ten days later, each plant, with the soil intact, was transplanted singly to autoclaved soil in a 6" clay pot. Both while in the cups and in the pots, the plants were arranged together on the greenhouse bench by treatments in a randomized block set-up. Fertilizing regimes were as described, and watering was done on a daily basis.

Five weeks after transplanting to the cups, the plants were harvested. Data on heights and fresh weights of roots, shoots and whole plants were taken. The root systems were indexed for galling on a 1-6 rating scheme. A value of 1 was given for 0 infection; 2 = 1-10% of the root system galled, 3 = 11-30% infection, 4 = 31-70% infection, 5 = 71-90% infection, and 6 = 91-100% infection.

Drench experiment 2. Due to poor growth of plants in the first drench experiment, the test was repeated with some changes. Each treatment was only replicated 6 times and the treatments consisted of 30 ml drench with 1/5 S, 1/10 S, 1/20 S and 1/40 S aqueous extracts of derris roots. Sterile distilled water served as control. Since new derris roots were not available, the extracts in this test were obtained from roots that had been in storage for longer than 4 weeks. The soils used were mixed with commercial potting medium in a 10:1 ratio by volume.

The inoculation procedure was modified. The seedlings (3 weeks old) were transferred singly into the styrofoam cups. Three days later each seedling was inoculated with 500 larvae of M. incognita. The inoculation holes were covered and the soil was drenched with the extract or distilled water.

When the plants were harvested at 5 weeks after inoculation, data on plant heights, fresh weights of roots, shoots and whole plants, and gall rating were taken. The dry weights of the plants were recorded after a 24-hour drying in the oven at 80°C.

Amendment experiments. Testings of derris roots for efficacy as soil amendment against M. incognita were repeated 3 times. The first two trials were with newly received roots (1 week in storage). Plants were grown in soils similar to those described in Drench experiment 1, without the addition of potting medium. Roots were incorporated into the soil in styrofoam cups at 0.2%, 0.5%, 1% and 2% dry weight equivalent to weight of soil. Control treatments received no amendment.

In the first experiment, crudely ground root materials were incubated in the soil for 2 weeks, then a 14-day old tomato seedling was planted in each cup. Each plant was then inoculated with 500 larvae of M. incognita.

The root materials in the second experiment were not ground, but were incorporated as small pieces cut and split into approximately 5 mm x 3 mm size. The materials were incubated in the soil for 1 month, then 2 weeks old tomato seedlings were planted singly in the soil in each cup. The plants were then inoculated with 500 M. incognita larvae.

A few modifications in procedure were made in the third experiment. The soil used was mixed with potting medium in a 10:1 ratio by volume. As new root materials were not available, roots that had been in storage for 8 weeks were used as amendment. The roots were cut into small pieces, soaked for 1 hour in water, and ground moist for 1 minute in a Laboratory blender. The crudely ground materials were mixed into 200 g soil in 200 ml styrofoam cups at the rate of 0.2%, 0.5%, 1% or 2% dry root equivalent by weight to the weight of the soil. Each amendment level was replicated 10 times. A set of 10 cups without the derris

amendment served as controls. The 50 cups were arranged on the greenhouse bench and the soil kept slightly moist.

Ten days after incorporation of the root materials, the soil in each cup was inoculated with 500 M. incognita larvae. Four days later a 3-week old tomato seedling was planted into each cup. After 10 days, the seedlings were each transplanted intact with the soil into a 6-inch clay pot containing 600 ml (660 g) autoclaved soil. The plants were arranged on the greenhouse bench in a randomized-block set-up both while in the cups and in the pots. Maintenance and growth conditions were as described for other pot experiments (Drench experiments).

The plants were harvested at 5 weeks after inoculation. Data on heights, fresh and dry (24-hour drying at 80°C) weights of shoots, roots and whole plants were recorded. Root systems were indexed for galling on a 1-6 evaluation scheme.

CHAPTER IV

RESULTS

Bioassays of Aqueous Extracts

Aqueous extracts of derris roots showed a low level toxicity to adults of A. avenae and C. elegans, and larvae of M. incognita. The nematodes were not visibly affected in the first 6 hours but were immobilized by extracts at up to 10-fold dilution within 24 hours (Table 1). In occasional checking where immobilized nematodes were transferred to water, M. incognita and A. avenae generally recovered from the effect of 24 hours immersion in stock extract solution but were killed by 48 hours exposure. At 20-fold dilution or more, the extracts did not immobilize these two nematode species but were still toxic to C. elegans. The activity of the extracts generally correlated with their concentrations. C. elegans was the most sensitive of the nematodes tested.

When the derris roots had been in storage for 8 weeks, the extracts were not toxic to A. avenae and M. incognita (Table 1). The high sensitivity of C. elegans was again demonstrated in this test. At the stock and 5-fold dilution levels a high number of the nematode were still immobilized in 24 hours and a 100% mortality was effected in 24 hours. The toxic activity was much reduced at lower strengths and was practically absent at 20-fold dilution. C. elegans individuals that were immobilized in 24 or 48 hours at all extract levels were usually killed.

TABLE 1. Activity of aqueous extracts of Derris elliptica roots against Caenorhabditis elegans, Meloidogyne incognita and Aphelenchus avenae in Immersion tests.^a

Nematodes	% number of nematodes immobilized in											
	Extracts of new roots ^b				Control	Extracts of aged roots ^c						
	S	1/5 S	1/10 S	1/20 S	1/40 S	(H ₂ O)	S	1/5 S	1/10 S	1/20 S	1/40 S	
<u>C. elegans</u>												
24 hr	100f	100f	100f	35c	0a	0a	92a	65d	15b	0a	0a	
48 hr	100e	100e	100e	85d	40c	5a	100e	100e	38c	10b	4a	
<u>M. incognita</u>												
24 hr	100c	100c	85b	0a	0a	0a	0a	0a	0a	0a	0a	
48 hr	100d	100d	100d	5b	0a	0a	25c	9b	0a	0a	0a	
<u>A. avenae</u>												
24 hr	100c	100c	55b	0a	0a	0a	0a	0a	0a	0a	0a	
48 hr	100c	100c	77b	0a	0a	0a	0a	0a	0a	0a	0a	

^aResults are means of 5 replications with 40-50 nematodes per replicate. Means in a row followed by the same letter are not significantly different at the 5% level as determined by Duncan's multiple range test.

^bRoots kept in storage for 1 week. Stock (S) extract was obtained by extracting 1 g root with 3 ml water. The others were prepared by dilution of the stock in water.

^cRoots kept in storage for 8 weeks. Stock (S) extract was obtained by extracting 1 g root with 3 ml water. The others were prepared by dilution of the stock in water.

Bioassays of Organic-Solvent Extracts

The derris resins obtained from extractions with organic solvents were not highly soluble in water and were redissolved in minimal amounts of acetone or ethanol. To maintain the solvents to a level not toxic to the nematodes, the resins were further diluted in sterile distilled water to the equivalent of 0.25 g root/ml water. The activity of the extracts against the test nematodes is presented in Table 2 below.

A. avenae and M. incognita were not affected by any of the extracts in 48 hours (Table 2). Benzene, methanol and chloroform extracts were toxic to a small number of C. elegans, but were not as active as acetone or ethanol extracts which killed 100% of the specimens

TABLE 2. Activity of organic-solvent extracts of Derris elliptica roots against Caenorhabditis elegans, Meloidogyne incognita and Aphelenchus avenae in an Immersion test.^a

Extracts ^b	% of nematodes immobilized					
	<u>C. elegans</u>		<u>M. incognita</u>		<u>A. avenae</u>	
	24 hr	48 hr	24 hr	48 hr	24 hr	28 hr
Acetone	100	100	0	0	0	0
Benzene	25	40	0	0	0	0
Chloroform	45	60	0	0	0	0
Ethanol	100	100	0	0	0	0
Methanol	50	60	0	0	0	0
Controls						
1% acetone	0	12	0	0	0	0
1% ethanol	0	10	0	0	0	0

^aResults are means of 4 replications, with 40-50 nematodes each.

^bEach extract was diluted in water to 0.25 g root/ml.

in 24 hours. In a separate side test using lower strengths, acetone extract was found to retain higher activity than ethanol extract. The choice of acetone for use as the solvent in extractions for fractionation purposes was based on the results of these tests.

Bioassays of Commercial Rotenone

Rotenone was moderately soluble in acetone. On adding water to dilute the solvent however, a significant proportion of the rotenone precipitated out in the solution after several hours. This was especially true with the higher rotenone levels. In the absence of a more suitable means of preparing the solution, saturated solutions (containing the rotenone) were used in the bioassays.

Rotenone at up to 500 $\mu\text{g}/\text{ml}$ caused no detectable effects on M. incognita or A. avenae in 72 hours (Table 3). C. elegans appeared to be slowed down in movements within 1 hour of immersion in 250 and 500 $\mu\text{g}/\text{ml}$ solutions, but obviously recovered later on. Individuals were not killed in 24 hours, and only 15% of the nematodes were dead after 48 hours in 500 $\mu\text{g}/\text{ml}$ rotenone. No indication of toxicity was observed at lower strengths. Since in preliminary observations, C. elegans activity after 48 hours in 1% acetone or 1% ethanol (which served as controls in this test) was often inconsistent, and unreliable as a basis for comparison, results with this nematode were recorded only for a 48-hour period.

TABLE 3. Effects of rotenone against Caenorhabditis elegans, Meloidogyne incognita and Aphelenchus avenae in an Immersion test.

Rotenone ($\mu\text{g/ml}$)	% nematode mortality ^a							
	<u>C. elegans</u>		<u>M. incognita</u>		<u>A. avenae</u>			
	24 hr	48 hr ^b	24 hr	48 hr	24 hr	48 hr	72 hr	72 hr
500	0	15	0	0	0	0	0	0
250	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0
Control (1% acetone)	0	0	0	0	0	0	0	0

^aResults are means of 5 replications, with 40-50 nematodes per replicate.

^bFigures were adjusted for nematode mortality in 1% acetone (about 10%).

Thin-Layer Chromatography

A preliminary work using Chloroform-acetic acid (199:1) as a solvent system did not yield satisfactory results with derris extracts. Improved separation was obtained by developing the chromatogram to 15 cm distance, but the development time for this was quite long (approximately 60 minutes). The benzene-acetone (9:2) system that was finally used gave a fair separation of the fractionates over a 15 cm development distance, for a development time of 35 minutes. Trials with several other combinations of different solvents indicated this solvent system as the most satisfactory.

At least 14 fractionates were detected on chromatograms of acetone extracts of derris root (Fig. 2). Seven of these were visible as yellow spots without chemical treatment of the plate. The others were detected on Silica Gel G after spraying with iodine solution or hydriodic acid, followed by heating. Potassium permanganate was less satisfactory as a detection reagent in this case as the spots, which were often very close together, all appeared white on a pink background.

Rotenone could not be detected on Silica Gel G without chemical reagent. Spraying with hydriodic acid produced three spots with a characteristic blue color. The main spot (based on size and color intensity) was located on the same band as fractionate number 3 of the acetone extract and this was considered to be that of rotenone. The other two spots are likely to be break-down products of rotenone.

Fig. 2. Chromatogram of acetone extract of Derris elliptica roots and rotenone standard (R) on Silica Gel G developed in benzene-acetone (9:2) and sprayed with hydriodic acid. The dotted spots show the positions of fractionates as they were collected for bioassays. Toxic fractionate 1, as determined from bioassays, is marked Toxic.



For bioassay purposes, the fractionates were collected in 15 bands to include the base line, according to spot positions and each about 1 cm wide. The relative positions of these fractionates are presented in Table 4.

Five spots were detected on chromatograms of aqueous extracts of derris. Bioassays of small amounts of collected fractionates did not yield positive results. In the course of the work, it was found that the aqueous extracts of available root samples had lost much of their activity with aging of the roots in storage. Due to the difficulty of

TABLE 4. Chromatographic features of acetone extract of derris roots on Silica Gel G developed in benzene-acetone (9:2) system.^a

Fractionate	Band position (cm)	r.f.	Spot detections
1	13.5 - 15	0.95	visible
2	12.5 - 13.5	0.86	chemical spray
3	11.3 - 12.5	0.79	visible
4	10.6 - 11.3	0.73	chemical spray
5	9.7 - 10.6	0.68	visible
6	8.8 - 9.7	0.62	chemical spray
7	7.4 - 8.8	0.54	visible
8	6.7 - 7.4	0.47	chemical spray
9	5.8 - 6.7	0.42	chemical spray
10	4.5 - 5.8	0.34	visible
11	3.5 - 4.5	0.27	chemical spray
12	2.5 - 3.5	0.20	visible
13	1.4 - 2.5	0.13	chemical spray
14	0 - 1.4	0.05	visible
(Base)	(Base Line)	0.00	-

^aChromatograms were developed for a 15-cm distance.

^bSpots not yielding visible colors were detected by spraying the plates with iodine solution or hydriodic acid, followed by heating.

collecting large amounts of fractionates from aqueous extract by chromatography, isolation attempts with this extract were not pursued more extensively. Furthermore, aqueous extracts of roots that had been extracted several times with acetone were very low in activity.

Bioassays of Acetone Extract Fractionates

The results of preliminary bioassays of acetone fractionates are shown in Table 5. Fractionates 3 and 4 which corresponded closely with standard rotenone in chromatographic positions showed no toxic activity to the nematode species tested. Fractionates 6 and 7 were slightly active on C. elegans but did not kill or immobilize M. incognita in 48 hours. When these fractionates were tested at half the concentrations, the activity against C. elegans did not persist. They were considered only mildly toxic and were not evaluated further. The most active fractionate in the test was fractionate 5. This is referred to as Toxic fractionate 1. The fractionate killed both C. elegans and larvae of M. incognita in 24 hours. Toxic fractionate 1 was further evaluated more closely.

Activity of Toxic Fractionate 1

In a further bioassay by Immersion test, Toxic fractionate 1 showed a high activity against C. elegans and M. incognita larvae. At 1000 ppm, the fractionate killed all specimens of both nematode in 24 hours (Table 6). With C. elegans the effects were more pronounced as 100% of the nematode were killed in 6 hours. The young adults of this

TABLE 5. Immersion test bioassay of fractionates from acetone extract of derris root against Caenorhabditis elegans and Meloidogyne incognita.

Fractionate	Test strength		% nematode mortality ^a				
	Actual (ppm)	Root equiv. (g/ml)	<u>C. elegans</u>		<u>M. elegans</u>		
			24 hr	48 hr	24 hr	48 hr	72 hr
1	1000	0.43	0	5	0	0	0
2	1000	0.91	0	10	0	0	0
3	2000	0.23	0	10	0	0	0
4	2000	0.75	0	7	0	0	0
5	1000	0.50	100	100	100	100	100
6	1000	0.81	25	55	0	0	11
7	2000	0.47	21	45	0	0	7
8	1000	0.62	0	13	0	0	0
9	1000	0.49	2	10	0	0	1
10	1500	0.45	5	15	0	0	1
11	1000	0.92	0	7	0	0	0
12	1000	0.53	0	5	0	0	0
13	1000	0.89	0	5	0	0	0
14	1000	0.82	0	11	0	0	2
Base	1000	0.37	0	15	0	0	0
Control	(Silica gel eluant in 1% acetone)		0	8	0	0	1

^aResults are means of 3 replications with 20-40 nematodes each.

TABLE 6. The activity of Toxic fractionate 1 from derris roots against young adults of Caenorhabditis elegans and Meloidogyne incognita larvae.^a

<u>Test strengths</u>		<u>% nematode mortality</u>				
Actual (ppm)	Root equivalent ^b (g/ml)	<u>C. elegans</u>		<u>M. incognita</u>		
		6 hr	24 hr	24 hr	48 hr	72 hr
1000	0.500	100	100	100	100	100
750	0.375	85	100	90	100	100
500	0.250	0	100	84	93	100
250	0.125	0	0	0	0	5
100	0.050	0	0	0	0	0
Control (1% acetone)		0	0	0	0	0

^aResults are means of 5 replications, with 40-50 nematodes each.

^bBased on 2000 µg of the fractionate obtained per gram of root in extractions.

species were almost stationary within 1 hour of immersion. At about 3 hours the nematode became active in situ with violent trembling of the body. This continued for about one-half hour, after which the nematode ceased visible movement. When checked by touching with a needle at 6 hours, the nematode did not respond.

The activity of Toxic fractionate 1 against C. elegans persisted at a lower level at 500 ppm (0.25 g root/ml) but was absent in a 24 hour immersion at 250 ppm. The activity against M. incognita was also high at 500 ppm crude fractionate, and practically disappeared at 250 ppm level. The effects of the fractionate at 1000 ppm were noticeable early. The larvae slowed down greatly within 2-3 hours. A small percentage of the nematode assumed a near twisted posture within 6 hours.

At 24 hours the larvae had the characteristic dead posture (Fig. 3); the body rigid and the internal cavity much vacuolated indicating disintegration of tissues. It would appear from the results that the active principle(s) in the fractionate is a fast-acting substance.

Effects of Toxic Fractionate 1 on Hatching

Toxic fractionate 1 at 1000 ppm, 750 ppm and 500 ppm greatly reduced larval emergence from M. incognita eggs in the 6 day test period, as compared to acetone 1% control (Fig. 4). The inhibition was greatest during the first two days of incubation and persisted for the 6 days. The total larval counts in 1000 ppm and 750 ppm solutions were each less than 20% of those in acetone. Hatching of eggs in 500 ppm was 7.4% total for the 6 days, which was about 35% of those in acetone control. Due to the diminishing amounts of the incubation solutions in the watch glasses, the evaluation of hatching in the fractionate solutions was terminated after 6 days. The eggs were further evaluated for hatching in water.

The hatching of the eggs in water was obviously influenced by the previous treatments of the eggs (Fig. 5). Eggs removed from 1% acetone solution (control) continued to give the highest number of larval hatch. Those previously in 1000 ppm yielded the lowest larval counts in the 4 days period in water. Hatching of eggs previously held in 750 ppm was similarly low. Eggs previously incubated in 500 ppm yielded about 50% of the larval count recorded for acetone control.

Fig. 3. Meloidogyne incognita larvae killed by Toxic fractionate 1 in an immersion test.



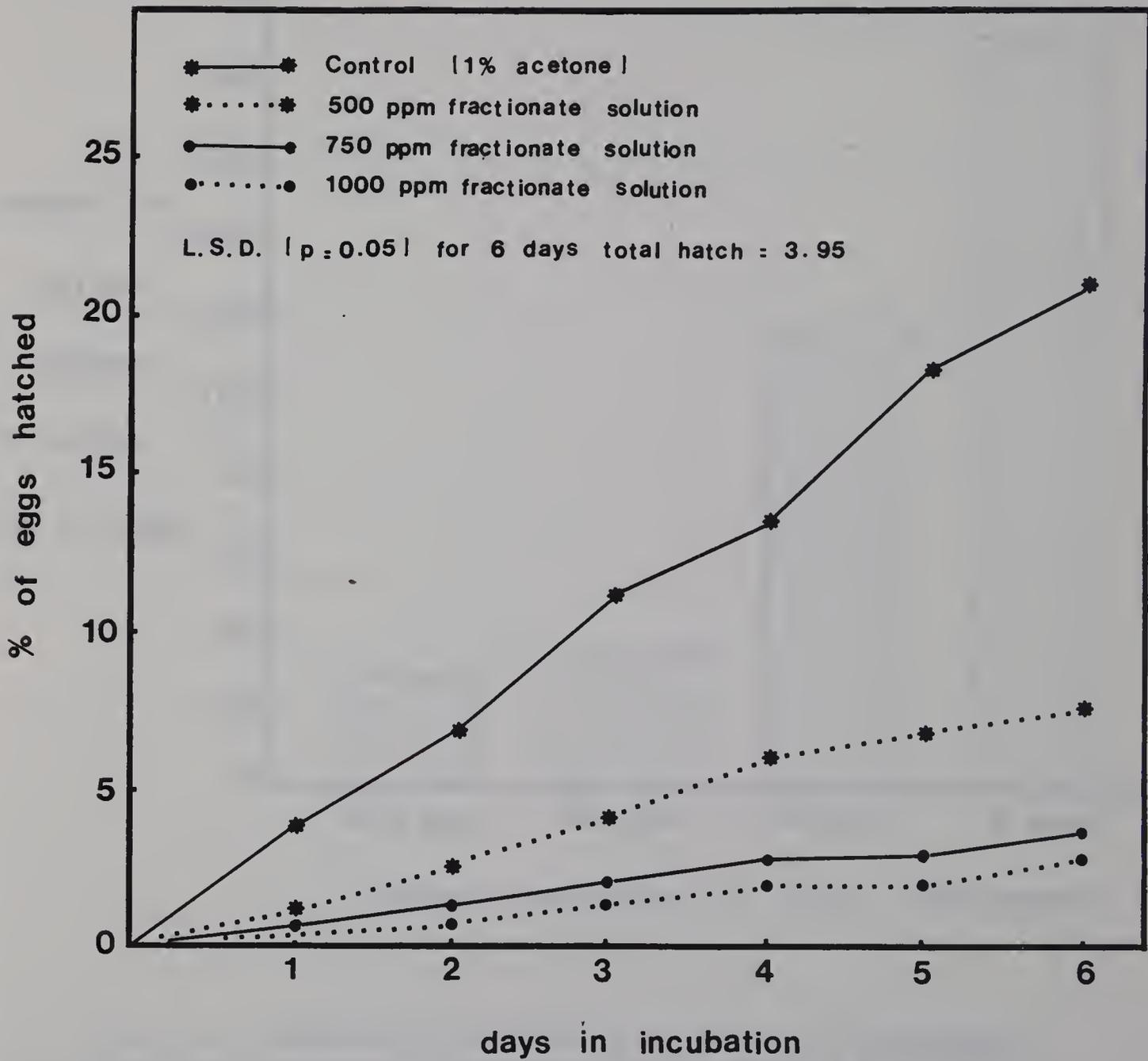


Fig. 4. Effects of Toxic fractionate 1 from derris root on larval hatch of incubated Meloidogyne incognita eggs.

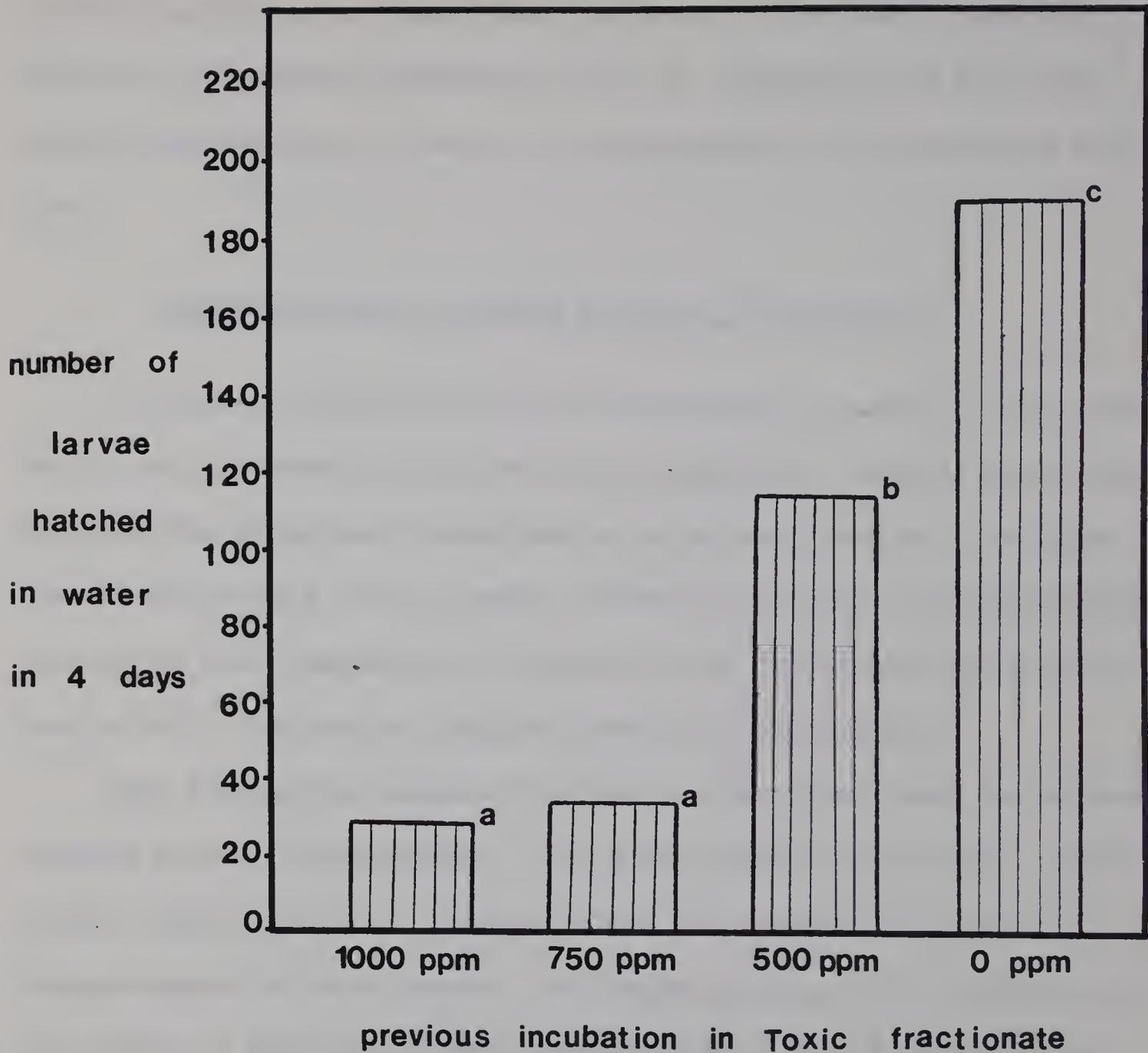


Fig. 5. Cumulative 4-day hatch in water of Meloidogyne incognita eggs previously incubated for 6 days in solutions of Toxic fractionate 1 or 1% acetone (0 ppm fractionate). Egg hatch represented by chart bars designated with similar letter are not significantly different at 1% level as determined by Duncan's multiple-range test.

Although the above evaluations could not be carried out for an extended period, it is clear that the Toxic fractionate 1 adversely affected the hatching physiology of the M. incognita eggs and these effects persisted after removal of the eggs from the fractionate solutions.

Characterization Attempts on Toxic Fractionate 1

A further evaluation of toxic fractionate 1, such as in pot experiments, was prevented by the absence of activity in similar fractionate obtained from subsequent isolations of other root samples. As fresh root materials were not available, effective isolation and characterization of the toxic component(s) present in the fractionate could not be carried out. The results obtained herein are preliminary.

Both fractionate samples from the non-toxic and toxic isolations produced similar chromatograms. The fractionates yielded one lightly visible yellow spot (r.f. 0.6) on Silica gel G developed in a 9:2 benzene-acetone solvent system. Following spraying with hydriodic acid and heating, 2 other spots were identified at r.f. 0.5 and 0.9. On plates developed in chloroform-acetic acid (199:1), the fractionates differentiated to 5 components (see Fig. 6). Three of these (r.f. 0.15, 0.3 and 0.65) were lightly visible as yellow spots. The other two (r.f. 0.55 and 0.9) were identified after spraying with hydriodic acid followed by heating. Rotenone, run as standard, differentiated into 3 spots which appeared blue immediately upon spraying with hydriodic acid. The main spot (r.f. 0.55) corresponded with spot number 3 of the

Fig. 6. Toxic fractionate 1 from toxic (1) and non-toxic (2) isolations differentiating into components on Silica Gel G following development in chloroform-acetic acid (199:1). R is rotenone standard.



fractionate both in color development and chromatographic position. The other 2 spots were at r.f. 0.67 and 0.3. These were in the same bands for spots number 2 and 4 of the fractionate respectively, but differed in color development. Spot number 3 of the fractionate is likely to be rotenone since the fractionates were collected from regions immediately below the band for rotenone in the acetone extract chromatograms.

The relative weights of the various components within the two fractionate samples differed. In the first sample (toxic isolation), the ratio by weights of components 1 through 5 were roughly 1:4:1:1:1. In the second sample, the proportions were 1:1:1:2:1 respectively. None of the components from both samples, including the base spots (base lines), were active on M. incognita or C. elegans in Immersion tests at 500 ppm concentrations.

Greenhouse Drench Experiments

In the first Drench experiment, the growth of tomato plants over an 8-week period was not satisfactory. The plants were generally stunted despite adequate fertilizing. This was probably due to the low soil pH and the low organic matter content of the soil. The effects of soil drench with aqueous extracts of new roots of derris on root-gall of the tomato were however clear cut (Table 7, Figure 7).

All levels of extract strengths reduced galling by M. incognita and most significantly at 5 fold (1/5 S) dilution (Table 7). The extracts caused a slight but insignificant reduction in root development at 5 fold dilution. At the full stock concentration, seedlings were

Fig. 7. Root-galling of tomato reduced by drenches with aqueous extracts of derris roots. S/5, S/10 and S/20 represent treatments with 5-fold, 10-fold and 20-fold dilutions of stock derris extract respectively. Control plant (0) was not treated.



TABLE 7. Effects of aqueous extracts of new derris roots^x on root-galling (Meloidogyne incognita) and growth of tomato plants.^y

Extract strengths	Plant ht (cm)	Shoot fresh wt (g)	Root fresh wt (g)	Root-gall index ^z
S (stock)	(dead)	-	-	-
1/5 S	6.85 a	2.02 a	0.69 a	2.7 a
1/10 S	7.35 b	3.31 b	1.25 b	3.6 b
1/20 S	6.87 a	1.97 a	0.85 ab	4.1 b
Control (H ₂ O)	6.14 c	1.53 a	0.91 a	5.5 c

^xRoots kept in storage for 1 week.

^yResults are means of 10 replications. Means in a column followed by the same letter are not significantly different from each other at the 5% level as determined by Duncan's multiple-range test.

^zEvaluated on a 1-6 scale: 1 = 0% of the root system galled; 2 = 1-10%; 3 = 11-30%; 4 = 31-70%; 5 = 71-90%; 6 = 91-100%.

killed within a few days. Plant heights and shoot development were not adversely affected, and in 10 fold and 5 fold dilutions treatments, were better than in controls. Extract at 10 fold dilution gave the best results in terms of plant growth.

Extracts from aged derris root (second drench test) did not cause significant reduction of root galling of tomato (Table 8). Plant growths were slightly retarded at 5 fold and 10 fold dilutions of the extracts.

Amendment Experiments

Tomato plants in the first two amendment experiments were generally stunted, again probably a reflection of the poor soil conditions.

TABLE 8. Effects of aqueous extracts of aged^w Derris elliptica roots on the growth and root-gall index (Meloidogyne incognita) of tomato.^x

Extract strengths ^y	Plant height (cm)	Fresh wt (g)		Dry wt (g)	Root-gall index ^z
		shoot	root	whole plant	
1/5 S	29.50 a	24.9 a	9.5 a	3.38 a	3.17 a
1/10 S	31.83 a	26.0 ab	8.9 a	3.55 a	3.50 a
1/20 S	33.00 a	31.5 b	11.1 a	4.27 ab	3.50 a
1/40 S	31.08 a	31.0 b	11.0 a	4.70 b	3.67 a
Control (H ₂ O)	32.17 a	31.2 b	10.9 a	4.50 b	3.50 a

^wRoots kept in storage for longer than 4 weeks.

^xResults are means of 6 replications. Means in a column followed by the same letter are not significantly different at the 5% level as determined by Duncan's multiple-range test.

^yS indicates stock strength. The tested strengths were obtained by a 5-fold, 10-fold, 20-fold and 40-fold dilution of the stock with water.

^zRated on a 1-6 scale: 1 = 0% of the root system galled; 6 = 91-100%.

The results obtained were however on a similar pattern as those recorded in the third experiment (Table 9), in which the plant growths were more satisfactory.

Derris root amendment reduced galling only slightly at the 2% level, and was not effective at lower rates. A clear cut effect of the amendment on plant growth was absent. There was a slight but insignificant reduction in root development with increasing levels of the amendment. This could have been as a result of phytotoxic effects of the derris root materials as in the drench test. It might also be due to direct physical obstruction of the roots by the amendment. It was

TABLE 9. Growth and root-gall index (*Meloidogyne incognita*) of tomato plants grown in soil amended with roots^X of *Derris elliptica*.^Y

Amendment level	Plant height (cm)	Fresh wt (g)		Dry wt (g) whole plant	Root-gall index ^Z
		shoot	root		
2%	31.8 a	31.20 a	7.82 a	4.10 a	2.8 a
1%	31.4 a	29.60 a	8.10 a	3.89 a	3.2 ab
0.5%	32.5 a	29.10 a	9.29 a	3.93 a	3.4 b
0.2%	31.4 a	29.03 a	8.31 a	3.76 a	3.5 b
0%	32.9 a	30.52 a	9.38 a	3.84 a	3.5 b

^XRoot materials were incubated in the soil for 2 weeks before the tomato were inoculated with the nematodes.

^YResults are means of 10 replications. Means in a column followed by the same letter are not significantly different at the 5% level as determined by Duncan's multiple-range test.

^ZRated on a 1-6 scale: 1 = 0% of the root system galled; 2 = 1-10%; 3 = 11-30%; 4 = 31-70%; 5 = 71-90%; 6 = 91-100%.

noticed that the higher amendment levels caused soil clumping, and pieces of the derris root materials stuck to the tomato roots.

C H A P T E R V

DISCUSSION

Aqueous Extracts

Derris roots contain water-extractable constituent(s) toxic to nematodes. The active component did not appear to be very stable. In the immersion tests, the reduction of toxicity in the second trial was likely to be due to degradation of the nematotoxic substance with aging of the roots in storage. Derris root materials generally do not keep their high toxicity to insects for longer than 10 days when exposed to sunlight (Pagan and Morris, 1953). Rotenone decomposes readily on chromatography plates if subjected to light (Cheng et al., 1972) and most of the break-down products are not toxic to insects.

Rotenone was not found lethal to nematodes in the present study. However, there is the possibility that one of the rotenoids present in derris root is nematotoxic. Little information is available on these substances regarding their stability. It seems probable that they degrade as easily as rotenone, in view that the insecticidal activity of the derris root (for which the rotenoids are partially responsible) is lost totally fast. In the current study, the immersion tests were carried out with minimal exposure to light. This did not preclude, however, possible degradation by air, to which rotenone is known to be sensitive (Gunther, 1943; Cheng et al., 1972).

Rotenone

For practical purposes rotenone cannot be considered nematocidal. The substance was ineffective even at very high concentrations. The low solubility of this substance in water further poses a problem in getting it to reach nematode targets in the soil. This probably contributed to its ineffectiveness in the immersion test as well. Murfitt et al. (1976) found this substance inhibitory to the stage-3 respiratory process of C. elegans. This study was conducted with a mitochondrial preparation, however, and not with intact nematodes. In the immersion test C. elegans slowed down during the first few hours but later recovered. The effect of rotenone was either low level and slow or only temporary. Rich et al. (1977) similarly did not find this substance lethal to Pratylenchus schribneri, although it caused a slight inhibition of the nematode movement. The effects on motility were not studied in the current work. It is not likely that this substance would be effective against nematodes in the soil.

Acetone Extract

The low activity shown by chloroform and benzene extracts suggests that the toxic component involved is a polar substance. This is supported by the higher activity of the acetone and ethanol extracts. There is a possibility that more than one nematotoxic component is present in the derris root. Throughout the study acetone extract was found to show fairly consistent activity against C. elegans, in contrast to the varied results obtained with the aqueous extract. This suggests that

one of the active components was relatively stable, and was more effectively extracted with acetone than water. The absence of activity of the acetone extract on M. incognita and A. avenae at the tested extract preparation level (0.25 g root/ml) could be due to low amount of this substance in the root. This was demonstrated in the activity of Toxic fractionate 1 obtained from acetone extract. The fractionate was not active at below 500 ppm (0.25 g root/ml).

The activity of the active component(s) from Toxic fractionate 1 could not be evaluated properly. There were 5 components detected from the fractionate. One of these is suggested to be rotenone, which was shown to be non-toxic to nematodes. The active component must be active at below 500 ppm, since the crude fractionate was toxic at this level. The activity of the substance is probably high, and its effects on the nematodes fast. Nematodes that were immobilized within 24 hours were all killed. In the hatching test, egg hatch was inhibited from the first day and this persisted throughout the 6 days. This suggests that the toxic substance was either lethal to the eggs or it maintained high activity in solution without degradation, or both. A sample of the fractionate solution stored at 5°C for more than 5 days retained high activity against nematodes. The continued low level hatch of the eggs after removal to water indicates that the effects of the toxic substance on the eggs were persistent at least for several days.

The reason for the lack of activity in the second fractionate isolation could not be pinpointed to an obvious cause. Similar varied results were obtained with another fractionate in earlier work involving

smaller amounts of materials. The positive results could not be confirmed in subsequent isolations. Results with Toxic fractionate 1 were however considered reliable since the relatively large amount of fractionate obtained in the toxic isolation allowed for more repetitions of bioassays. It is felt that the present investigation was hampered by unavailability of roots in fresh condition. The roots that were received from Brunei had aged at least 3 weeks from the time they were harvested before they could be used. Although attempts were made to use only the best root specimens in the batch, it was likely that some chemical degradation had set in. Furthermore, the roots did not all come from the same plant, and were obviously of different sizes and ages. The degradation rates in such roots would vary.

The second possibility was that the toxic component was present in a smaller amount in the second fractionate preparation than in the first, due to different concentrations of the substance in the roots. This was likely as the proportions of the 5 components in the 2 fractionates differed. Unfortunately this could not be confirmed as the toxic component could not be isolated by rechromatography of the toxic fractionate sample. Although the toxic component appeared to be stable in solution, the additional exposure incurred in air-drying and rechromatography apparently degraded the substance.

Greenhouse Bioassays

The results obtained with the derris materials in the greenhouse pot experiments were a general reflection of those observed in

laboratory assays. Drenching with aqueous extracts from new roots was effective in reducing root-galling by M. incognita, whereas similar extracts from roots aged in storage were not. This correlated with observations on the activity of the extracts in the Immersion tests. It would appear that in the first drench experiment, the aqueous extract killed or had long-term adverse effects on M. incognita larvae in the soil as the treatment was only applied once. The ineffectiveness of extracts from aged roots indicates that degradation of toxicity had set in. Although the extracts were still moderately active in vitro, the toxicity was further reduced in the soil.

The aqueous extracts of derris were evidently phytotoxic at high concentrations, as seen in the death of seedlings in stock extract treatments. Similarly, the higher extract levels reduced plant growth in the second experiment. In the first experiment, these effects were not evident on the surviving plants, since the low root-galling at the higher extract strengths were in marked contrast to the heavy nematode infection on the control plants. These results seem promising. Although the extracts are phytotoxic, injury to plants could be avoided by drenching planting sites several days ahead of planting. The high extract strength for the drench treatment to be effective is an obvious limitation, but its use may be feasible in small scale topical applications.

The low effects of the amendment treatments on root infection by M. incognita probably accrued from degradation of toxic components in the soil. It is possible that better nematode control would have been

achieved if the nematode inoculum was introduced into the soil prior to or immediately after incorporation of the root materials. Apparently, this would require very high amendment levels, since as evidenced from the drench experiments, high extract concentrations were necessary for effective nematode control. With amendment, the problem would be more acute. The toxic components conceivably are released slowly and the apparently high degradation rate of the roots does not allow for sufficient accumulative effects to develop against the nematodes. Based on these considerations the best means for using the derris materials in crude form seems to be as aqueous extracts applied before planting.

Results obtained in the current investigation suggest some possibility of using derris root as a nematode control material on limited applications. An interesting development may accrue with further work on the toxic fractionate as isolated from acetone extract of the roots. The current work was hampered by unavailability of fresh roots with which a more conclusive characterization of the toxic factor in the root would have been possible. It is felt that the toxic component may be present in the root in small quantity, but that the substance seems to be strongly active. Although derris root may not be used directly as a control material, identification of the toxic component would enable synthesis of analogues with similar activity. Further work with fresh root materials is warranted.

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