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IN VITRO CULTURING OF THE BURROWING NEMATODE, *RADOPHOLUS SIMILIS*, A SERIOUS ROOT PEST OF TEA, ON TISSUE CULTURE RAISED FROM CARROT AND GINGER

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Plant parasitic nematodes attacking tea, especially in the mid and lower elevation tea areas, generally occur as concomitant species and consequently it is difficult to obtain pure populations of nematodes for precise detailed investigations. Attempts were therefore made to mass culture the burrowing nematode, *Radopholus similis* collected from tea areas on callus tissues grown from sliced discs of carrot and ginger. Of the two, carrot tissues seemed to be the one suitable as large populations amounting to about 15,000 - 20,000 pure nematodes were obtained from an initial inoculum level of only 25 nematodes within a short span of 60 days from inoculation. Though ginger is known to be a good host to this species of nematode, attempts to mass culture *R. similis* on excised discs of ginger was unsuccessful. This limitation appears to be due to some nematicidal substance released from excised ginger tissue. Experiments with diluted water extracts of ginger revealed that there was good correlation between increasing death rate of nematode with slight increases in added diluted ginger-water extract.

Large populations of uncontaminated nematodes obtained by such *in vitro* culturing methods on carrot tissue culture have now enabled us to carry out several detailed investigations such as screening for resistance; screening for the presence of bio-types and investigate breakdown of resistance; *in vitro* screening of nematicidal properties of natural plant extracts; establishing economic damage threshold levels; interrelationship with other organisms, etc.

INTRODUCTION

In general, a given dominant species of plant parasitic nematode exists concomitantly along with one or more other species of parasitic nematodes, within the rhizosphere of a given host plant. Such groups of parasitic nematodes that are usually associated together on the same host plant, are referred to as "concomitant species". Besides different species of nematodes being associated together, there is also a different association in the rhizosphere with other micro-organisms, such as fungi and bacteria, which also influence the performance, growth and productivity of the particular host plant. Such a complex situation makes it extremely difficult for the nematologists to study in precise detail the host/parasitic relationship between a particular host plant and a given species of nematode in a specific environment. The need for using pure cultures of nematodes, therefore, is important for such precise economic studies. Pure cultures of nematodes are also essential for a range of other important studies, including: investigations on the biochemical basis of resistance of plants to nematode infestation; studies on disease complexes; studies involving the assessment of pheromone activity; to rear predators and parasites of nematodes for biological control work; and for studies to evolve methods of control at the physiological level, using metabolic disruptors.

Such a precise technique of study of the behaviour and influence of a single species of organism in the absence of other species is known as 'Gnotobiology'. This technique has been adopted by Mycologists and Microbiologists for nearly 100 years (Koening and Barker, 1985).

Byars (1914) was the first nematologist to culture plant parasitic nematodes monoaxenically*. He sterilised the egg masses of *Meloidogyne* sp and inoculated them onto the seedlings of tomato and cowpea which were grown in Pfeffers nutrient agar medium. By this technique, he was able to describe in detail the life cycle of the root-knot nematode. Tyler (1933) and Hastings and Boscher (1938) also separately developed successful methods of sterilizing nematodes for inoculating seedlings grown in test tubes. Tyler thus was able to demonstrate parthenogenesis in root-knot nematode.

However, significant progress was achieved only in 1955, after Mountain developed a method of rearing *Pratylenchus minyus* on sterile excised corn roots. Thereafter, several nematologists have worked on this technique and such information is available in reviews written by Zuckerman (1969, 1971), Vanfleteren (1978) and Koening and Barker (1985).

Although *in vitro* culturing of specific insect species have been successfully developed on oligidic, meridic and holidic media at the Tea Research Institute of Sri Lanka (Gnanapragasam, 1979, 1983), attempts to rear plant parasitic nematodes of tea was started only recently as 1987. This became necessary with the spread and escalation of nematode problems in the mid and lower elevations, warranting detailed studies on the burrowing nematode, *Radopholus similis* which generally occurred as concomitant species with either *P. loosi* and/or *Rotylenchulus reniformis.*

Initial attempts were made to culture *R. similis* on callus tissue grown from sliced carrots (discs). Attempts were also made to rear this species of nematode on ginger which is a good host. Ginger in Sri Lanka is cheaper than carrots and readily available in the market. Ginger can also be bought in bulk and kept stored fresh for long time by burying in soil.

MATERIALS AND METHODS

Experiment 1 *In vitro* culturing of *R. similis* on carrot tissue

The laboratory method to mass rear *R. similis* on carrot tissue, is based on the technique developed by O'Bannon and Taylor in 1968, Koshy and Sossama (1980) and Huettel (1985) with minor modifications.

Fresh unbruised carrots (*Daucus carota* L) with intact above ground leaves are chosen for such studies. Carrots stored in the refrigerator are avoided as these are more prone to bacterial and fungal infection (Moody, Lownsberry and Ahmed, 1973; Huettel, 1985). These chosen carrots were thoroughly scrubbed in clean water to remove dirt and dipped in 95% ethanol. Thereafter, they were flamed and peeled under sterile conditions, using a laminar flow-hood, and sliced into one cm thick discs. Using sterile forceps, a disc each was transferred onto 1% water-agar slants in culture tubes measuring 15 X 150 mm or onto 1 % water-agar contained in 150 ml conical flasks. The tubes and the flasks were plugged with sterile cotton wool and left in a room maintained

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* Monoaxenic culture: a medium containing two species, one being the plant and the other the nematode

at a constant temperature of 25° ± 1°C, 75 % ± 2 % RH until callus formation. The initiation of the callus occurred in 4-6 days under our laboratory conditions (Fig. 1). In order to obtain maximum number of nematodes per culture, we have assessed the optimum time for inoculation to be one week after initiation of callus i.e. about two weeks from the time of placing the carrot discs onto the agar medium. If inoculation was carried out too early, the population build-up was found to be very slow.

Experiment II *In vitro* culturing *on R. similis on ginger tissue culture*

Fresh succulent ginger was used for this study. Ginger was surface sterilised as above (Experiment 1) peeled and sliced under sterile conditions under the "laminar flow hood". The sliced ginger was then placed on 1 % water-agar medium contained in tubes measuring 15 X 150 mm or in 150 ml conical flasks.

The initiation of callusing of ginger occurred in 2-3 days time under our laboratory conditions (25° ± 1°C and 75% ± 2% RH). Inoculation was carried out 3 days after placing the ginger slices on agar medium, which was found to be the optimum time for inoculation on ginger discs.

Inoculation

Nematodes recovered from the roots of tea or from the soil in the rhizosphere of tea plants by the Baerman funnel technique were individually picked and placed in sterile water contained in a sterile BPI dish. These isolated nematodes were then surface sterilised in the following manner with the aid of a centrifuge run at 3000 rpm (a) fifteen seconds in sterile water; (b) one minute in 0.01% mercuric chloride solution; (c) three washings in sterile water; (d) fifteen seconds in 0.5 streptomycin solution and (e) two rinses in sterile water. A drop of sterile water containing about 25 surface sterilised nematodes is then placed on the callusing carrot or ginger discs with the aid of a sterile pipette under the laminar flow-hood. The inoculated flasks and tubes are then stored in an incubator maintained at 25° C ± 1° C (Fig. 2).

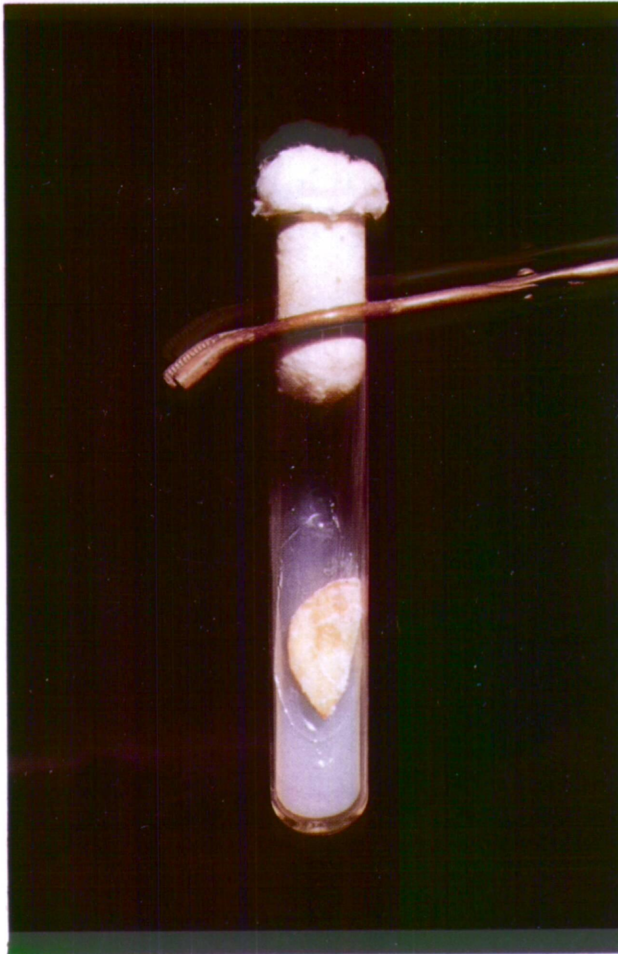

Fig. 1 — *Callussed carrot tissue on a water-agar slant contained in a sterile culture tube measuring 15 X 150 mm plugged with sterile cotton wool*

(a)

(b)

Fig. 2 — *Calussed carrot tissues inoculated with sterile Radopholus similis contained in (a) sterile culture tubes measuring 15X150 mm and (b) sterile conical flasks of capacity 150 ml, maintained in an incubator at 25 ± 1° C*

RESULTS AND DISCUSSION

Experiment I

Periodical observations made at 3-day intervals revealed a significant build-up of populations on carrot discs (400 fold increase), after a period of 39 days following inoculation. However, the maximum build-up (700-800 fold) was observed between 60-70 days from inoculation. Beyond this period there was a decline in population and large numbers of dead nematodes were observed after about 78 days, and the carrot discs were observed to become shrivelled.

The several thousands of pure cultures of nematodes within each culture tube can easily be sub-cultured thereafter, to build-up a very large number, for several laboratory studies.

Using this technique it has now become possible to obtain a population of around 15,000 – 20,000 uncontaminated nematodes from an initial population of only 25 nematodes within a short span of 60 days.

Experiment II

Periodical observations made at 3-day intervals revealed a build-up of population only up to about 20 days. The maximum number that could be obtained was only about 100-150 (4-6 fold increase) which number remained steady up to about 70 days. Beyond this period the ginger discs started rotting.

Despite being a good host to this species of nematodes, the failure for a rapid population build-up in sliced ginger is indicative of the possibility of some detrimental chemical changes occurring when the ginger was sliced which changes seemed to inhibit population build-up.

Another interesting observation was made during the process of extracting the nematodes (*R. similis*) from the ginger culture. When the ginger and the agar pieces were sliced and kept for few hours to collect the nematodes, although live nematodes were obtained after 5 h these died after a period of 12 h. However, if few ml of water was added to the culture tubes or flasks and the nematodes allowed to migrate into the water (without chopping the ginger disc) they remained alive even after a period of 24 h. In order to confirm the production of any nematicidal chemical property that may have been produced from the sliced ginger, the following experiment was carried out.

Experiment III Effect of water-extract of ginger tissue on the mortality of *Radopholus similis.*

Fifty nematodes of the same age were added to five small glass vials each containing 10 ml of sterile water into which different concentrations (ranging from 1 to 10 drops) of water-extract of ginger tissue (5 g of ginger tissue macerated with 50 ml of water) were added. As a comparison water extracts of carrot tissue of comparable strengths were added to similar number of glass vials inoculated with 50 nematodes each. A similar number of vials containing 10 ml of sterile water inoculated with 50 nematodes each were also left as untreated control.

Observations were made at 8, 12 and 24 h from introducing the nematodes into the tubes by counting the number of dead and live nematodes. The results are presented in Table 1.

TABLE 1 – *Survival of Radopholus similis in water extracts of ginger and carrot*

Treatments	*Period of observation*								
	8 h			12 h			24 h		
	Alive	Dead	% Dead	Alive	Dead	% Dead	Alive	Dead	% Dead
1 drop of ginger extract 10 ml $^{-1}$ water	47	3	(6)	38	12	(24)	22	28	(56)
3 drops of ginger extract 10 ml $^{-1}$ water	42	8	(16)	29	21	(42)	25	25	(50)
5 drops of ginger extract 10 ml $^{-1}$ water	40	10	(20)	27	23	(46)	17	33	(66)
10 drops of ginger extract 10 ml $^{-1}$ water	36	14	(28)	22	28	(56)	12	38	(76)
1 drop of carrot extract 10 ml $^{-1}$ water	50	0	(0)	50	0	(0)	47	03	(6)
3 drops of carrot extract 10 ml $^{-1}$ water	47	3	(6)	47	3	(6)	45	05	(10)
5 drops of carrot extract 10 ml $^{-1}$ water	47	3	(6)	45	5	(10)	44	6	(12)
10 drops of carrot extract 10 ml $^{-1}$ water	43	7	(14)	42	8	(16)	38	12	(24)
Sterile water	50	0	(0)	49	1	(2)	47	3	(6)

As seen in Table there was a linear increase in death of nematodes with increase in concentration and time in the tubes where ginger extract has been added indicating some chemical with nematicidal activity present in sliced ginger. There was hardly any death of nematodes in tubes treated with either the extracts of carrot tissue or the water control.

CONCLUSION

The pathogenic species of plant parasitic nematodes present in the mid and lower elevation tea areas generally occur as concomitant species and as such in the past one of the limitations that existed in carrying out detailed investigations on the burrowing nematode, *Radopholus similis* was the difficulties encountered in obtaining large numbers of pure populations of this species of nematode.

A technique has now been perfected to mass culture *R. similis* on carrot callus tissue generating about 15,000 - 20,000 uncontaminated nematodes within a short span of 60 days from inoculation.

Attempts to mass culture this species of nematode on ginger callus tissue, on the other hand, was not successful, possibly due to release of certain toxic chemical substances from the ginger tissue, as evident from the preliminary observations. Further investigations are warranted to determine the chemical nature of this nematicidal substance in ginger.

The technique to mass culture pure populations of *Radopholus similis* in laboratory culture has now enabled us to carry out several lines of precise and detailed investigations including screening of tea clones for susceptibility/resistance/tolerance to this species of nematode; screening for resistance of other crops grown in tea areas; screening for the presence of bio-types of different plant pathogenic species and investigate breakdown of resistance of some clones; *in vitro* screening of nematicidal properties of natural plant extracts; establishing economic damage threshold levels; interrelationship of nematodes with other organisms, etc.

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