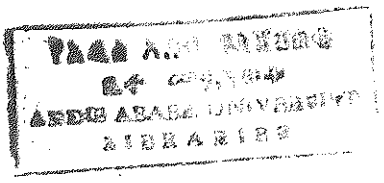
CHARACTERIZATION OF TWO NEMATODE-DESTROYING FUNGI FROM ETHIOPIA

THEODROS BEDELU



A Thesis Submitted in (part) Fulfilment For the Degree of Master of Science in Biology in Addis Ababa University

Addis Ababa

June, 1997

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ACKNOWLEDGEMENTS

I would like to extend my deepest gratitude to my advisor Dr. Dawit Abate for the suggestions and constructive crticisms he made during the progress of this work, and the literature he provided. I am also gratetful to Ato Amare Gessesse without whose help and critical supervision the enzymology part of the work could not have been undertaken.

I wish to thank Prof. Frau H. Anke (Unversity of Kaiserslautern, Department of Biotechnology, Germany) for providing the literature related with this work, Dr Kifle Dagne for taking pictures of the fungus-trapped nematodes, Ato Wondirad Mandefro for providing seeds of the tomato cultivar Rutgers and pertinent literature, and Dr Masresha Fetene for the greenhouse space he made available.

I finally thank the Swedish Agency for Research Cooperation with Developing Countries (SAREC) for finacial support of this work.

ABSTRACT

Two nematode-trapping *Arthrobotrys* spp., coded ART-1 & ART-2, were isolated from soil samples collected in Ethiopia.

Under laboratory conditions, ART-1 (LD_{50}= 290 conidia/ml) was more efficient in destroying nematodes than ART-2 (LD_{50}= 725 conidia/ml). Investigation on the growth of *Meloidogyne incognita*-infected tomatoes in the greenhouse showed that yield loss was highest in tomatoes treated with only *M. incognita* (69.7%). Tomatoes treated with conidia of ART-1 or ART-2 had lower losses: 44.5% for ART-1 treated & 44% for ART-2 treated tomatoes. ART-2 required 3X the number of conidia of ART-1 (180000 conidia/pot) to attain a nematode suppression effect similar to ART-1.

Out of 3 solid media compared for their effects on the lateral growths of the isolates at room temperature, oatmeal agar (OA) gave the greatest colony diameters (6.3 cm for ART-1 & 7.8 cm for ART-2). Maximal spore yields were also observed on this medium: ART-1, 4960 conidia/ml & ART-2, 31400 conidia/ml. A comparison of lateral growth, on medium A (MA), under different incubation temperatures (room temperature, 28°C, 32°C, & 37°C) showed that greatest colony diameters were attained at 28°C: 5 cm & 5.7 cm for ART-1 & ART-2 respectively.

Broth of MA was found to be suitable for optimal mycelial biomass yield for both isolates (631 mg/100 ml, ART-1; 755 mg/100 ml, ART-2). However, ART-1, a slow grower on all solid media tested, gave a higher yield than ART-2 when grown in casein mineral salts medium 1 (CMS-1) broth (363 mg/100 ml Cf. 278 mg/100 ml). Optimal mycelial biomass yield in MA broth was found to be in the pH range 4-5 for ART-2 (509-669 mg/ 100 ml) & in the range 7-8 for ART-1 (487-535 mg/100 ml).

ART-1 & ART-2 produced extracellular proteases in CMS-2 broth. The proteases from both fungi had a temperature optimum of 50°C, & were optimally active in the alkaline range (7.5-8, ART-1 & 7.5-9, ART-2). ART-1 produced more proteases (19 U/ml/hr) than ART-2 (14 U/ml/hr). ART-1 proteases were more efficient in degrading nematodes than those of ART-2 by virtue of being produced in a larger quantity, suggesting that the difference in the nematode-destroying capabilities of ART-1 & ART-2 was the result of the difference in the amount of proteases produced.

1. INTRODUCTION

Nematodes are one of the important pathogens of plants. Because most plant-parasitic nematodes attack plant roots, nematode-caused plant damage is often unnoticed (Mai, 1985).

Nematode problems are more pronounced in tropical and subtropical agriculture (Mai, 1985; Luc *et al.*, 1990). Plant-parasitic nematodes in the tropics have shorter life cycles. This results in a more rapid population explosion than in the temperate areas. For example in the temperate areas, the cyst nematode *Heterodera* sp. produces upto two generations per year, whereas the same genus in the tropics produces a generation every 25 days.(Luc *et al.*,1990 The higher temperatures and longer growing seasons in the tropics encourage a buildup of higher nematode populations and lead to more crop damage.

In the tropics a crop is often attacked by a number of damaging nematode species. Sugar cane can be damaged by 10-20 species of genera such as *Meloidogyne*, *Heterodera*, *Pratylenchus*, and *Xiphinema* (Luc *et al.*, 1990). Although there are secondary nematode species that attack a crop in temperate areas, more often there is only one parasite of a crop that can easily be managed by control efforts.

In the irrigated tropical lowlands the practice of monoculture, continuous cropping, non-crop rotation, and the use of homogenous germplasm create favourable conditions for the high incidence of plant-parasitic nematodes. Under such conditions the incidence of more damaging species like *Meloidogyne incognita* increases.

The Ethiopian nematode situation is not different from the above observations. In his survey of Ethiopian crops, O'Bannon (1975) has identified twenty one genera of plant-parasitic nematodes. Members of the genus *Melodogyne* (root-knot nematodes) were found in association with nearly 40% of the crops sampled. Table 1, taken from O'Bannon, gives a list of some nematode genera and the crops with which they are associated.

Root-knot nematodes are known to parasitize more than 200 plants species including almost all cultivated plants (Agrios, 1978). Coffee, cotton, tomato, and onion are among the cultivated, economically important crops and vegetables that are attacked by root-knot nematodes in Ethiopia. So far the only yield loss assessment study, made in this country, on the fibre crop kenaf (*Hibiscus canabinus*) has shown that root-knot nematodes cause considerable damage: a 46% loss has been recorded at Melkawerer (IAR, 1985).

Yield loss in nematode infected plants is a result of lowered efficiency of roots in absorbing water and nutrient elements, and increased susceptibility to the attack of microbial pathogens. Nematode-fungus disease complexes are well recognised. *Fusarium* wilt of plants increases in severity when the plants are also infected with nematodes (Moorman *et al.*, 1981). The same holds true for the bacterial wilt of tobacco; many plant viruses are also transmitted through nematode vectors (Agrios, 1978; Mai, 1985).

To control plant-parasitic nematodes, cultural, chemical, physical, resistant varieties and biological methods are used. Cultural methods like crop rotation and fallowing can reduce nematode incidence. However, cultural methods are of little practical use when one considers growers' reluctance to cultivate less economically important crops in place of the more

important but nematode- susceptible ones, or , far worse, to leave arable land uncultivated (Jatala, 1985). Chemical control methods are increasingly losing their appeal because of their adverse effects on man and the environment. Physical control methods such as heat treatment of soil and planting material are too expensive to be of practical use. The development of nematode-resistant plant varieties takes a very long period of time. Biological control, on the other hand, is thought to offer natural regulation of pest populations and thus has little or no adverse effect on the environment. Much of recent research concerned with alternative nematode control methods is directed towards biological control (Stirling, 1991).

The biological control of plant parasitic nematodes mainly involves the manipulation of populations of nematode-antagonistic organisms resident in soil. Many soil-inhabiting microorganisms are known to be nematode-antagonistic. Fungi are one of them. More than 100 fungal species attack nematodes (Stadler *et al.*, 1993a).

The potential of some of the fungi to be used as biological control agents is highly promising. *Paecilomyces lilacinus*, an egg parasite of root-knot nematodes, is a case in point. Tests with this fungus have shown successful results in Argentina, Ecuador, Panama, Peru, and the USA (Jatala, 1985).

Natural fungal enemies of root-knot nematodes from the rhizosphere of plants have not been investigated in Etiopia. Thus the objectives of this work were:

1. To isolate the natural fungal antagonists of nematodes.
2. To test the effectiveness of the fungi on free-living nematodes *in vitro*.
3. To characterize the promising fungal isolates.

4. To determine the optimum growth conditions of the isolates.

5. To investigate the production, characteristics, and biological significance of proteases from the isolates in submerged culture.

6. To determine the effect of the isolates on *M. incognita* infected tomatoes in experimental pots in the greenhouse.

Table 1. The major plant-parasitic genera found in association with various hosts in Ethiopia (O'Bannon,1975).

Nematode genera	Host association
1. *Ditylenchus*	chickpea, sweet potato
2 Helicotylenchus	coffee, maize, banana, *Eragrostis teff,* sorghum, haricot bean
3. *Heterodera*	maize, tree tomato, banana, citrus, cotton,
4. *Hoplolaimus*	maize, *Phytolaca dedicantra*
5. *Meloidogyne*	coffee, cotton, banana, onion, soybean, alfalfa, *Enste ventricosum*, chickpea, endod, lettuce, tomato, sunflower, grape
6. *Radopholus*	banana
7. Rotylenchus	coffee, maize, sorghum, *Enste ventricosum*, barley, citrus, haricot bean
8. *Pratylenchus*	*Eragrostis teff*, coffee, maize, wheat, barley, *Enste ventricosum*

Table 2. Genera of nematophagous fungi and the plant-parasitic nematodes they attack (Gray, 1987; Stirling, 1991).

Fungal genera	Nematode genera
1. *Catenaria*	*Xiphinema*
2. *Arthrobotrys*	*Meloidogyne*, *Tylenchorhynchus*, *Pratylenchus*, *Xiphinema*, and *Hoplolaimus*
3. *Hirsutella*	*Criconemella*, *Heterodera*
4. *Paecilomyces*	eggs of *Meloidogyne* and *Heterodera*
5. *Verticillium*	in cysts or eggs of *Globodera*, *Heterodera*, and *Meloidogyne*
6. *Dactylella*	eggs of *Meloidogyne*
7. *Harposporium*	*Aphelenchoids*

2. LITERATURE REVIEW

2.1. Plant-Parasitic Nematodes

Nematodes have worm-like morphological appearance. Their place in the taxonomic hierarchy of organisms has been a point of dispute. Until the late 1970's, they were regarded as a class (Class Nematoda) under Phylum or Subphylum Nemathelminthes (Steiner, 1960; Goodey, 1963; Agrios, 1978). More recently, however, they have been elevated to a separate phylum: Phylum Nematoda (Dropkin, 1980; Pechenik, 1985). In both cases plant parasitic nematodes are placed under the orders Tylenchida and Dorylaimida, the majority being in the former.

Based on the type of parasitism, plant parasitic-nematodes can be classified as ecto-, semiendo-, and endo-parasites (Jones and Jones, 1977). Ectoparasites feed on young root tips and root hairs. Notable among them: *Xiphinema* spp. (dagger nematodes) and *Pratylenchus* spp. (pin nematodes). They cause root-tip gall formation and distortions. Semiendoparsites spend their feeding time by partly embedding the frontal parts of their bodies in host roots. Lance (*Hoplolaimus* spp.), spiral (*Rotylinchus* spp.) and stunt (*Tylenchorhynchus* spp.) nematodes are common forms of this group. Endoparasites spend most part of their life cycle in plant root tissues. Typical examples include root-knot, burrowing (*Radopholus* spp.) and cyst nematodes. Female root-knot nematodes lose their motility and become pear shaped inside roots, causing tissue swelling (galling). The burrowing nematode is a well known banana root pathogen; it causes browning and cracking of banana roots. Females of *Heterodera* spp. lay a large number of eggs; when a female stops laying eggs, the remaining eggs together with second stage larvae are retained inside her body. The body wall then gets

hardened by extrasecretion of cuticle. It develops into a cyst wall capable of resisting moisture stress.

2.2. Nematophagous Fungi

2.2.1. The nematophagous habit

Diverse forms of soil-inhabiting fungi feed on nematodes. They include species belonging to the Ascomycetes, Basidiomycetes, Deuteromycetes and Zygomycetes (Mankau, 1980). They are given the collective name nematophagous or nematode-destroying fungi. They have undergone sophisticated structural modifications for the purpose of infecting or capturing nematodes.

Nematode-destroying fungi could be endoparasites, predatory, female or egg parasites in habit. At times, intermediate forms between endoparasitic and predatory fungi are recognized. The Basidiomycete genus *Nematoctonus* is a case in point (Thorn and Barron, 1984). Table 2, taken from Gray (1987) and Stirling (1991) , lists some nematophagous fungi and their nematode hosts.

Nematode endoparasitic fungi exist almost for the entire part of their life cycles inside the bodies of their hosts. Outside the host they produce no vegetative mycelia but are found mainly as conidia. Often obligate parasites, they are rarely maintained on artificial media under laboratory conditions.

Three groups of nematode endoparasitic fungi are recognized on the basis of their infective structures: endoparasites with encysting zoospores, endoparasites with adhesive spores, and endoparasites with ingested conidia.

Endoparasites with encysting zoospore have flagellated zoospores that serve as infective units. The zoospores develop into cysts when they attach themselves to body openings (i.e, anus, vulva, buccal cavity) of a nematode host. Host penetration is achieved by means of germ tubes. Members of the genera *Catenaria* (Chitridiomycetes) and of *Myzocytium* (Oomycetes) are typical examples of endoparasites producing encysting spores (Gray, 1987).

Endoparasites with adhesive spores produce conidia, that are covered with a sticky, adhesive substance at one end. The conidia stick to host cuticle with the help of the adhesive material. Germ-tube mediated penetration follows after attachment. Notable genera with adhesive spores include: the Deuteromycetes *Drechmeria* (Dijksterhius *et al.*, 1991) and *Verticillium* (Durschner-Peltz and Atkinson, 1988); the Basidiomycete *Nematoctonus* (Stirling, 1991); and the Deuteromycete *Hirsutella* (Jaffee, 1992).

In the case of endoparasitic fungi with ingested conidia nematode infection is initiated when the conidia, eaten by the host, start germinating inside the buccal cavity or gut of the host. So far ingested conidia have only been known from the genus *Harposporium* (Stirling, 1991).

Predatory fungi capture and feed on nematodes with modified mycelial structures. In recent litrature the confusing phrase 'predatory fungi'is replaced by the more precise 'nematode-trapping fungi'(Stirling, 1991). Most predatory fungi have adhesive traps serving as capturing organs. A few others have toxin-producing cells along their mycelia.

Generally, six different types of mycelial, adhesive traps are recognized among predaceous fungi: undifferentiated adhesive hyphae, adhesive branches, adhesive nets, adhesive knobs, constricting rings, and nonconistricting rings. Fig. 1, taken from Gray (1987), shows a diagramatic depiction of the various forms.

Undifferentiated adhesive hyphae are the least complex trapping organs. The entire or partial mycelial surfaces of fungi with this kind of trap are covered with a yellow colored, strongly adhesive secretion. Nematodes are immobilized 1 or 2 hours after contact with the hyphae. The Zygomycetes *Stylopage* and *Cystopage* and some Hyphomycetes (e.g. *Arthrobotrys botryospora*) capture nematodes by means of such hyphae (Gray, 1987; Stirling, 1991).

Adhesive branches are more complex than undifferentiated hyphae. Erect mycelial branches made up of 3-5 cells form, by anastomosing, arch-like adhesive loops or two dimensional networks. They are found closely spaced along a mycelium. The surface of each branch is covered with a thin layer of adhesive secretion. *Dactylella acruata* (Scheuner and Webster, 1990) and some members of the genus *Monacrosporium* (Domsch *et al.*, 1980) are typical examples of fungi producing adhesive branches.

Adhesive nets are believed to have evolved from adhesive branches. The complex three-dimensional adhesive nets are the most common type of traps observed in nematode-trapping fungi. Their formation is the result of the curving and fusion of erect, lateral mycelial branches with the parental hypha. The process is repeated until adhesive-covered, three-dimensional network of anastomosed loops is formed. The Hyphomycetes *Arthrobotrys*

oligospora, *A. conoides*, *A. arthrobotryoides*, and *A. superba*, are known to form adhesive networks (Domsch *et al.*, 1980; Stadler *et al.*, 1993a).

Adhhesive knobs are commonly globose, as in some members of the genus *Dactylella* (Stadler *et al.*, 1993a), or sometimes hourglass-shaped, as in the genus *Nematoctonus*, the imperfect state of the mushroom *Hohenbuehelia*(Barron and Dierkes, 1977; Thorn and Barron, 1984). The knobs are covered with adhesive material that firmly attaches them to nematodes at the time of contact.

Ring traps are made up of three or four crved cells supported by a two- or three-celled stalk arising from the parental hypha. Two forms of ringed traps are known: constricting and nonconstricting rings.

Constricting rings consist of three curved cells, supported on a short two-celled stalk, formed from an erect lateral mycelial branch. They are the most complex of all fungal trapping organs. Nematodes are held immobile, upon entering the rings, as a result of the rapid inward swelling of the cells. By so doing, the swollen cells exert a constricting force on the nematodes.

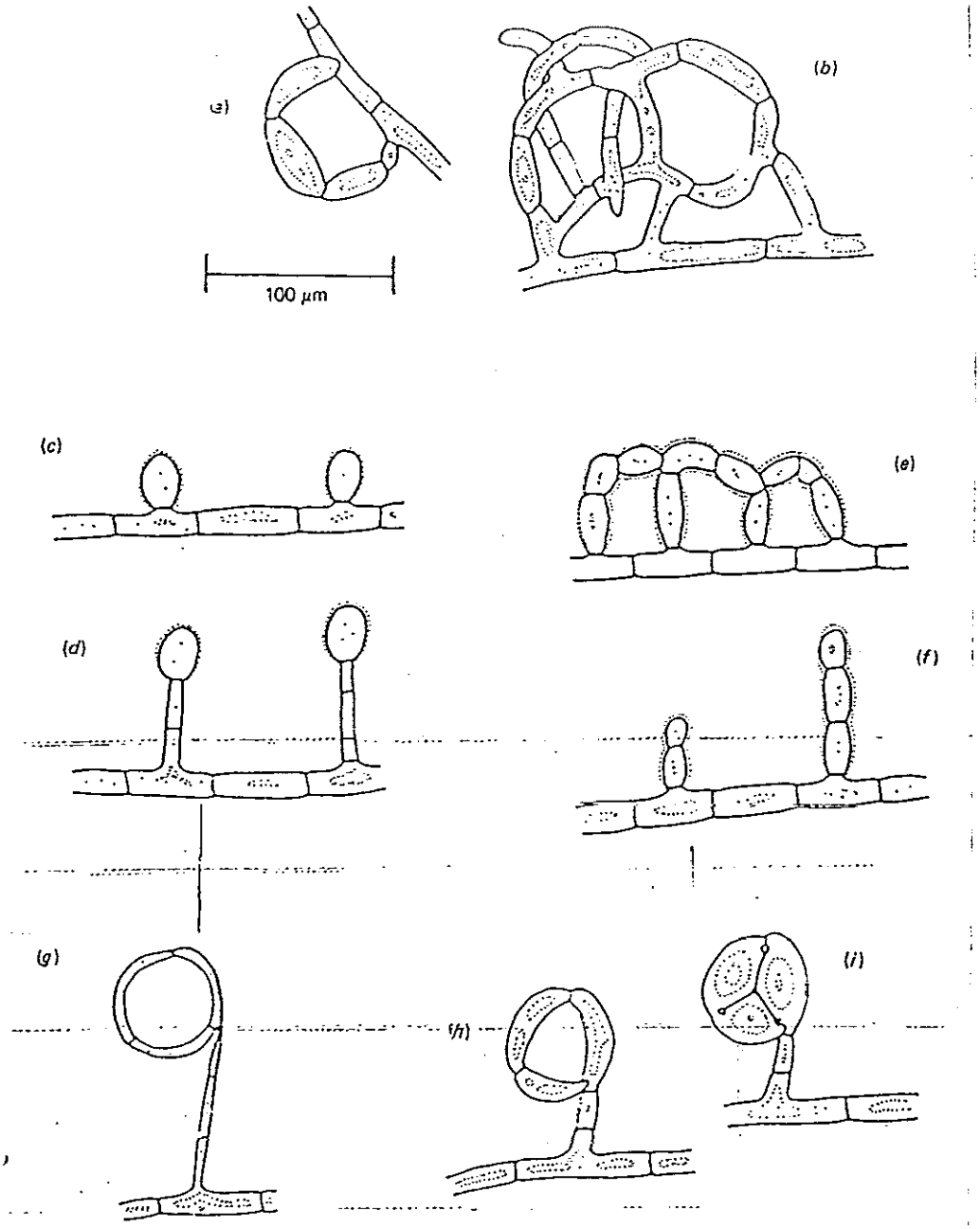


Fig. 1. Trapping organs of nematode-trapping fungi: adhesive nets forming simple (a) & (b) complex 3-dimensional traps; sessile (c) & stalked (d) adhesive knobs; & adhesive branches (f) often forming 2-dimensional adhesive net-works (e); nonconstricting rings (g) & consticting rings open (h) & closed (i) (Gray, 1987).

Higgens and Pramer (1967) have shown that electrical stimulation, increased temperature, and the action of touch can have the same ring cell swelling effect, on constricting ring forming fungus *A. dactyloides* , as nematodes. According to ultrastructural studies on the rings of *A. dactyloides* (Heinz and Pramer, 1972), cytoplasmic changes within the ring cells take place when the open configuration of the rings changes to the closed form. The most notable change being: the disappearance of electron-dense bodies located on the lumen side of the cells.

Nonconstricting rings, on the other hand, are made up of three or four curved cells supported by a three-celled, long mycelial stalk. Four species belonging to the genus *Dactylella* are known to produce them (Gray, 1987).

Since they cannot exert a constricting force, fungi with this kind of trap immobilize nematodes with the help of an adhesive layer that lines the insides of the rings (Dowsett and Reid, 1979).

There is a seperate group of predaceous fungi in which the usual trapping organs are lacking. Such fungi are able to immobilize and destroy nematodes through a different mechanism. The fungi were first noticed by Thorn and Barron (1984) when they were testing wood-rotting fungi for their ability to attack and consume nematodes. Four species of the mushroom *Pleurotus* were observed to exhibit the predaceous habit.

In a subsequent work, Barron and Thorn (1987) described the mechanism used by the mushroom *Pleurotus ostreatus* to attack its nematode victims. The mycelia of the fungus produce toxin droplets from minute spatulate secretory cells arrange ofd at irregular intervals. Upon contact with the droplets, nematodes are immobilized but not killed. Leakages from an

immobilized nematode induce directional hyphae to colonize the host through its body orifices, usually through the buccal cavity.

Among the flagellated zoospore producing endoparsitic fungi, there are a few that specifically attack sedentary female nematodes. Of these, *Catenaria auxiliaris* and *Nematophthora gynophila* are relatively well studied (Tribe, 1977a,b; Stirling, 1991). Both parasitize the females of the cyst forming nematode *Heterodera*.

The discovery of and interest in fungi parasitic on nematode eggs is nearly two decades old. Eggs of root-knot nematodes are known to be parasitized by *Dactylella oviparasitica* and *Paecilomyces lilacinus* (Stirling and Mankau, 1978; Caballinas *et al.*, 1989a). Records of *Verticillium chlamydosporium* occuring on eggs of cyst and root-knot nematodes are numerous (Stirling, 1991).

2.2.2. Physiological considerations

A. Chemotaxis

The mycelia of nematophagous fungi are capable of attracting nematodes. Monson *et al.* (1973) were the first to demonstrate the chemical nature of the attraction system. They were able to partially characterize the nematode attraction substance(s) (NAS) from culture filtrates of *Arthrobotrys fusiformis*. However, it was Jansson and Nordbring-Hertz (1979) who devised methods to investigate the attraction of nematodes to living mycelia of nematophagous fungi. They tested 14 fungi out of which 10 attracted, one (*A. arthrobotryoides*) repelled, and three

were neutral to the nematode *Panagrellus redivivus*. They further noted that the attraction intensity was directly proportional to the dependence of the fungi on the nematodes for nutrients.

The attraction towards the mycelia of nematophagous fungi can be influenced by the feeding habits of nematodes themselves. Mycophagous nematodes, for example, are readily attracted and captured by nematode-trapping fungi (Monson, 1968; Gray, 1987).

Conidia of nematode-endoparasitic fungi are also capable of nematode attraction. The observation that conidia of the fungus *Meria coniospora* infect *P. redivivus* at chemoreceptory sites (i.e., mouth and tail regions) (Jansson and Nordbring-hertz,1983), led to a subsequent work which demonstrated the significance of sialic acid (N-acetylneuraminic acid), located in the chemorecptory sites, in nematode chemotaxis and infection. Attraction of *P. redivivus* towards conidia was reduced when the nematodes were treated with the enzyme sialidase (neuraminidase) (Jansson and Nordbring-Hertz, 1984).

Even the nonattracting conidia of *Harposporium*-like endoparasites have attracting mycelia. Nematodes attracted by the mycelia accidentally ingest the conidia with other food substances (Gray, 1987).

The attraction process is completely reversed in endoparasitic fungi belonging to Chytridiomycetes and Oomycetes. Their motile zoospores are believed to find a host by moving along a chemical gradient formed by exudates released from nematode body orifices. Such response of zoospores to concentration gradients, however, has not been proven (Stirling,1991).

B. Mode of action

I. Attachment

Attachment of fungal infective units to attracted nematodes initiates the invasion process. Endoparasites with flagellated zoospores or ingested conidia face little difficulty in getting into the nematode internal system: they invade through nematode body orifices.

Endoparasites with adhesive conidia and predaceous fungi, on the other hand, have to contend with the nematode cuticular barrier. They have to maintain firm attachment to cuticle. Predators ensure this by using their adhesive trapping organs, and endoparasites with their adhesive conidia.

Adhesion is the result of interactions between complementary molecular configurations on nematode and fungal surfaces. Traps of *Arthrobotrys oligospora*, for example, possess lectin which binds to a carbohydrate on nematode surface (Nordbring-Hertz and Mattiasson, 1979). Preexposure of the traps to N-acetylgalactoseamine has been observed to result in failure to capture nematodes. Lectin-carbohydrate interactions result in firm binding of the fungus to the host nematode. Tunlid *et al.* (1992) have pointed out that the existence of similar processes in other nematophagous fungi is highly possible.

II. Enzymes, toxins, and antibiotics

The next stage in the infection process is penetration through the nematode cuticle. It is believed to be the result of both fungal enzymatic action and physical exertion. Reviewing

earlier scanning electron microscopic studies of the penetration process, Dijksterhuis *et al.* (1991) mentioned that the dual enzymatic-physical action was noted in *Drechmeria coniospora* and *A. oligospora*.

Nematode cuticle mainly consists of collagen secreted by the epidermis (Pechenik, 1985). Schenk *et al.* (1980) have detected and partially characterized extracellular collagenases from few nematode-trapping fungi. So far, collagenases produced by nematophagous fungi have been characterized as basic serine proteases: *A. oligospora* produces basic serine proteases in liquid cultures (Tunlid and Jansson, 1991); so does the nematode egg parsasite *Paecilomyces lilacinus* (Bonants *et al.*, 1995).

As early as the beginning of the 1960's, it was postulated that toxins are involved in the killing of nematodes after penetration (Olthof and Estey, 1963). Thirty years later linoleic acid was isolated for the first time and shown to be the only detectable nematicidal agent from submerged cultures of six Hyphomycete predaceous fungi (Stadler *et al.*, 1993a). Linoleic acid production by these fungi was found to be directly correlated to the number of traps formed.

Subsequent attempts to isolate nematicidal metabolites from cultures of other nematophagous fungi have met with success. Submerged cultures of the Basidiomycete *Pleurotus pulmonaries* yielded the nematicidal compounds S-coriolic acid, p-anisaldehyde, p-anisyl alcohol, 1-(4-methoxyphenyl)-1,2-propanediol, and 2-hydroxy-(4-methoxy)-propiophenol in addition to linoleic acid (Stadler *et al.*, 1993b). Linoleic acid was also isolated from the Ascomycete *Chlorosplenium* sp. (Anke *et al.*, 1995).

Other nematicidal metabolites from nematophagous fungi include: lachnumon and lachnumol A from the Ascomycete *Lachnum papyraceum* (Stadler *et al.*, 1993c; Stadler *et al.*, 1995b,c,d); and five sesquiterpenoids from the Basidiomycete *Cheimonophyllum candidissium* (Stadler *et al.*, 1994a).

Nematophagous fungi produce antibiotics inside the body of the nematode they have invaded. Antibiotic production is believed to be a competitve advantage that prevents other soil microorganisms from utilizing the captured nematodes as food source (Gray, 1987).

The isolation, characterization, and structural elucidations of antibiotics from nematophagous fungi have been given due attention in recent investigations. Three metabolites with antibacterial, antifungal, and cytotoxic activities have been reported from the Basidiomycete *Nematoctonus robustus* (Stadler *et al.*, 1994b). Six sesquiterpenoids with weak antifungal and antibacterial activities were isolated from the Basidiomycete *Cheimophyllum candidissium* (Stadler *et al.*, 1994a). Lachnumon and lachnumol A were also shown to have antimicrobial properties (Stadler *et al.*, 1993c). *Lachnum papyraceum* produces isocoumarin derivatives with weak antimicrobial activities (Stadler *et al.*, 1995a,b). Structural elucidations of the isocoumarins have been provided by Stadler *et al.* (1995c,d,). Submerged cultures of the

Hyphomycete *Arthrobotrys oligospora* have also yielded three antimicrobial metabolites: oligosporon (1), oligosporol A (2), and oligosporol B (2) (Stadler *et al.*, 1993b).

C. Trap induction

Nematode-trapping Hyphomycetes rarely form trap organs in pure cultures. Although spontaneous formation is sometimes observed (Nordbring-Hertz, 1974; Jansson and Nordbring-Hertz, 1979), trap organ production is usually initiated in the presence of nematodes. Nutrient level and environmental conditions also influence trap formation. Nordbring-Hertz (1968) has noticed the greatest number of trap organs on neutral pH and low C- and N- media that were seeded with nematodes.

Evidence supporting the assumption that a morphogenic substance produced by nematodes is responsible for trap induction led to the isolation of the substance from nematodes (Pramer and Stoll, 1959). Earlier attempts to characterize this substance, which was given the trivial name nemin, came up with the open-ended conclusion that it is a peptide of low molecular weight, or, possibly an amino acid (Winkler *et al.*, 1961; Pramer and Kuyama, 1963).

Noting earlier investigations have neglected the influence of environmental conditions on trap formation, Nordbring-Hertz (1973) showed that morphogenic substances can also be isolated from nutrient elements used for the growth of the fungi. Peptides from hydrolyzed casein were found to have inducing effect when added to cornmeal agar supporting the growth of *Arthrobotrys oligospora*. Partial characterization revealed the peptides responsible contained the amino acid residues leucine, isoleucine, valine, proline, and tyrosine (Nordbring-Hertz, 1974). Less frequently observed residues were: phenyl-alanine, glycine and alanine. In recent works, it has become a common practice to add dipeptides (usually phe-val) to broth cultures in order to induce trap formation (Stadler *et al.*, 1993a).

Trap induction in predaceous Hyphomycetes could also be influenced by bacteria. Rucker and Zacharia (1987) have noted induction by the nematode *Panagrellus silusia* in *Dactylaria brocophaga* and *A. conoides* was strongly influenced by several bacterial species.

Traps are usually produced by vegetative mycelia. However, conidial trap induction has been observed under certain conditions. Dackman and Nordbring-Hertz (1992) have reported that conidia of *Arthrobotrys oligospora* form traps when placed close to cow faeces on water agar plates. Diffusing substances from cow faeces are thought to induce the response. Conidial trap-forming mutant of this fungus has been obtained by a process analogous to microcycle conidiation (Nordbring-Hertz et al., 1995). The process involves encouraging trap formation by incubating conidia of the parent strain at an increased temperature (40°C) before incubating at the optimal 20°C.

2.2.3. Ecological considerations

The question of the extent to which nematophagous fungi are dependent on nematodes for their nutrition has got important implications. The question is more relevant when one considers the potential of the fungi as biocontrol agents.

Stirling (1991) regards the nutritional dependence of nematode-trapping (predaceous) fungi on nematodes as a continuum, with largely predaceous species and usually saprophytic species making up two extremes of the spectrum. This concept has been expanded, to include all nematophagous fungi, by replacing the phrases "largely predaceous" and "usually saprophytic" with "obligately parasitic" and "facultatively prasitic" respectively (Jaffee, 1992; Jaffee *et al.*, 1996).

Reviewing earlier nutritional studies on nematode-trapping fungi, Gray (1987) stated that although they utilized a wide range of of C- and N- sources in pure cultures, there were differences in the ability of some of the predatory Hyphomycetes to utilize various C- and N-sources. Constricting ring formers were found nutritionally much less versatile and more nematode-dependent than net formers. Among the experimental evidence cited: the net-forming *A. oligospora*, with high saprophytic ability, utilized all carbohydrates tested including polysaccharides like cellulose, starch and glycogen; the constricting ring former *Dactylaria bembicodes*, however, was unable to utilize cellulose, starch, or glycogen. The implication being: some net-forming species exist as saprophytes in soil even if nematodes are available for capture.

Quinn (1987) provided evidence in support of Cooke's (1968) hypothesis that the predaceous habit of nematode-trapping fungi is an adaptive mechanism to overcome nutritional limitations resulting from competition with other microbial organisms in soil. He noted that the nematode-trapping activity of *Arthrobotrys oligospora* and *Meria coniospora* increased when grown with saprophytic competitors (bacteria, fungi) in an artifitial soil substrate. Stirling (1991), however, points out that the differences in nematode populations between treatments of Quinn's experiments were relatively small and inconsistent to arrive at such a conclusion. After reevaluating Cooke's data, Gray (1987) concluded that the hypothesis may be applicable to the more saprophytic, net foming fungi than to more nematode-dependent, adhesive branch forming fungi.

The involvement of nematode-trapping fungi in food chain of cultivated or uncultivated soils is difficult to prove. Recently, Jaffee *et al.* (1996) attempted to show that nematode-trapping fungi supress insect-parasitic nematodes in a natural shrubland. Though they have isolated 11

species of predaceous fungi and obtained a significant *in vitro* nematode supression, their field tests showed that nematode supression was not correlated with fungal population density. However, they cautioned against rejecting the involvement of the fungi in food chain because existing methodologies for quantifying the numbers and activities of the fungi are inadequate.

Nematode endoparasitic fungi are often found on the obligately parasitic end of the nutritional spectrum. They are, to a large extent, dependent on nematodes. Their growth, if at all they grow, in pure cultures is limited. Their nematophagous activity is dependent on nematode population density. This has been shown for the endoparasite *Hirsutella rhossiliensis* (Jaffee, 1992).

There are even some obligate nematode egg parasites. *Dactylella oviparasitica*, the root-knot nematode egg parasite, is such an example (Stirling and Mankau, 1978). It is so dependent on nematode eggs that it has a growth rate of 1-2 mm/day on agar media.

2.3. Methods of Nematode Control

The aim of any pest management practice is to maintain the pest population below a level that causes economically undesirable plant yield loss. The control methods mentioned below, except for biological control, have been commonly used in nematode pest management. The use of a combination of two or more of the above control strategies constitutes integrated pest management.(IPM), which is more effective than any of them used alone.

A. Chemical

Chemical nematicides are highly effective in the control of plant parasitic nematodes. Results of trials conducted in Ethiopia have showen reduced nematode infections and higher yields of host plants.

The nematicides used include Phenamiphos on the banana cultivar Dwarf Cavendish; Aldicarb and Ethoprophos on the banana cultivar Poyo (IAR, 1983a; IAR, 1984). Corm treatment of the bananas with the nematicides was the application method.

However, it has been found that nematicides pose a threat to humans and other vertebrates. The nematicide DBCP has been found to contribute to the low fertility of workers in a factory manufacturing it; aldicarb was identified as a pollutant of ground water in New York (Johnson, 1985).

Studies on the toxicological effects of chemical nematicides on mammals underline the risks involved. Bloody nasal discharges, decreased body weight, and fetal weight loss have been observed in female rabbits administered with as little as 0.3 mg/kg phenamiphos (FAO, 1985). Ethoprophos adversely affects the reproduction of rats (FAO, 1983).

B. Physical and cultural

Heat treatment of soil and planting material is a common physical control method. Steam sterilization of greenhouse soil has been a long established practice; immersion of banana

planting part in water at 55^0C has been a commercial practice (Southy, 1965; Gowen and Queneherve, 1990).

Heat and other physical treatment methods (i.e. irradiation, electric current, etc.) are too expensive to be of practical use in large scale agriculture. And in the treatment of planting material, it is often difficult to maintain the critical balance between the intensity that is lethal to nematodes in the plant tissue and the one causing damage to the plant.

Cultural practices like crop rotation, fallowing, and time of planting reduce nematode populations. In an experiment to test the efficiency of dry fallowing for the control of *Meloidogyne* spp., a nine month period showed a greater population reduction than a three month fallow period (IAR, 1983b).

Jatala (1985) pointed out that economic pressure on land use is a constraint for the use of cultural practices, particularly in the case of fallowing and crop rotation.

C. Resistant varieties

There are plant varieties resistant to the attack of parasitic nematodes. It has been the task of plant breeders to develop varieties resistant to nematodes. Resistant cultivars show greater performance than susceptible ones in nematode-infested fields. Sasser and Kirby (1979) have made a list of more than 150 cultivars resistant to root-knot nematodes.

Resistant cultivars, however, do not retain this important trait for a prolonged period of time. They cannot be used in monoculture continuously. And it takes a longer period of time to develop new resistant cultivars (Jatala, 1985).

D. Biological

The limitations of the control methods mentioned above have forced researchers to look for alternative strategies that have little or no adverse effects on the environment. Biological control has become an appealing alternative.

According to Cook and Baker (1983), biological control is "the reduction of the amount of inoculum or disease producing activity of a pathogen accomplished by or through one or more organisms other than man." In the case of plant-parasitic nematodes reduction is believed to be achieved through the introduction or stimulation of their antagonists in soil.

Soil-inhabiting nematode enemies include, among others, micro-arthropods (eg. mites), protozoa, fungi, and bacteria. So far more than 100 fungal, seven bacterial, eight predatory micro-arthropods and one protozoan species have been recorded as nematode antagonists (Stirling, 1991).

2.4 Biocontrol Potential of Nematophagous Fungi

2.4.1. Nematode-trapping fungi

It has now been more than half a century since attempts to control nematodes by introducing nematode-trapping fungi have started. They were given attention because of their spectacular predaceous activity on agar plates (Mankau, 1980). However, a summary of the results of the various trials indicates that the performance of the fungi is inconsistent (Stirling, 1991).

Various reasons have been stated for the inconsistent performance of the fungi. Jatala (1985) considers the action of such fungi on nematodes as being passive and accidental, therefore less effective. But the validity of this explanation is questionable when it is examined in the light of the fact that the fungi have evolved a system of attracting nematodes. Another line of reasoning holds that predaceous fungi differ in their ability to capture nematodes (Sayre and Walker, 1991). Net-forming species, for instance, lead more of a saprophytic existence in soil, and thus have less regulatory effect on soil nematode population. Based on the ability to form traps, Jansson and Nordbring-Hertz (1979) divided nematode-trapping fungi into spontaneous trap formers (STF) and nonspontaneous trap formers (NSTF). STF include, according to Gray (1987), species that have enough energy in their conidia to produce traps spontaneously upon germination into mycelia. They are dependent on nematodes for their nutrition, and therefore have a more suppressive effect on nematodes. And yet there are some NSTF, for all their saprophytic nature, that have successful control on nematodes. Royal 300, a commercial name for the NSTF *Arthrobotrys robusta*, is a good example (Mankau, 1980).

In some of the trials that resulted in success, nematode suppression, upon revaluation, was attributed to the decomposition effects of organic amendments added rather than to the fungi (Sayre and Walker, 1991).

Perhaps the most likely explanation for the failure of nematode-trapping fungi to offer a consistent control is the fact that there is a lack of coincidence between trapping activity and the presence of nematodes in soil (Stirling, 1991). Most of the trials have been conducted against the root-knot and cyst forming nematodes which spend the most part of their life cycles in plant roots. Their free living, juvenile stages stay in soil, for a short time, only until they find a new host plant.

It should also be noted that the well known soil fungistatic effects (Watson and Ford, 1972; Lockwood, 1977) could have prevented germination of spores in those expriments in which conidia were used as inocula.

Still, the possibility for the successful utilization of these fungi exists. Conidial trap induction studies on *Arthrobotrys oligospora* (Dackman and Nordbring-Hertz, 1992; Nordbring-Hertz et al., 1995) have shown a possible way of improving the survival and trapping activity of predaceous fungi in soil. Conidial traps are regarded as survival stuctures to overcome fungistasis. Dackman and Nordbring-Hertz (1992) attributed the reduction of animal parasitic nematodes in the expriments of Gronvold et al., cited in Dackman and Nordbring-Hertz (1992), to the increased survival of conidia in cattle dung. Encouraged by this finding, researchers at the Commonwealth Scientific and Industrial Research Organization have screened over 2000 dung samples to isolate effective nematode-trapping fungi that could be used as control agents against livestock worm parasites (Spore, 1996).

2.4.2. Endoparasitic fungi

Because they are dependent on nematodes, endoparasitic fungi are more likely to be used as biocontrol agents in the future. However there are problems associated with their utilization.

The obligate nature of the fungi makes *in vitro* culturing very difficult (Jatala, 1986). Thus the problem of their mass production has yet to be solved. Even if mass production were possible there are difficulties of establishing the fungi in soil. Work with *Hirsutella rhossiliensis* has shown that the fungus requires some minimum number of nematodes (the host threshold density) to exist in soil; and that natural epidemics of the fungus develop slowly and only after long periods of high host density (Jaffee, 1992). Fungistatic effects may also limit the usefulness of these fungi (Stirling, 1991).

2.4.3. Egg and female parasites

The egg parasite *Paecilomyces lilacinus* has been the subject of interest in many a recent investigation concerning the biocontrol utilization of egg and female parasite of nematodes. This essentially saprophytic fungus (Domschet *et al.*, 1980) is known to aggressively colonize nematode eggs (Stirling, 1991). It has been found to be effective against root-knot and cyst nematodes (Jatala, 1985). Because of its apparent success further studies concerning its formulation, effective inoculum level, optimal time of application, temperature requirements, and histopathological interactions with root-knot nematode eggs have been made (Cabanillas *et al.*,1988; Cabanillas *et al.*, 1989a,b; Cabanillas and Barker, 1989).

However, in his assesment of the tests conducted using *P. lilacinus*, Stirling (1991) pointed out that many of the "experiments lacked treatments that enabled the effects of the fungus to be separated from those of the substrate on which it was grown." It has now become an

established fact that the addition of organic matter to soil in the range of of 0.5-1% w/w provides significant nematode control. Another problem with this fungus is that it is a human pathogen (Cabanillas and Barker, 1989). Eye infections and facial lesions can be caused by it.

Fungi that parasitize female nematodes are facultative soil saprophytes that are primarily nematode egg parasites. *Verticillium chlamydosporium* is a case in point (Jatala, 1986). Their parasitism of females is occassional and often not of much consequence.

3. MATERIALS AND METHODS

3.1. Isolation and maintenance of test organisms

The root-knot nematode *M. incognita* was obtained from Ato Wondirad Mandefro of the IAR, Ambo. It was maintained by transferring infected, chopped roots of the susceptible tomato cultivar Rutgers to pots cotaining three week old seedlings of the same cultivar. The inoculated seedlings could be kept in this manner for six to 10 weeks.

The bacteria feeding nematode, identified as being close to the genus level as c. f. (close to) *Protorhabiditis* by reference to Goodey (1963), was isolated from organic matter rich soil collected in Addis Ababa. The sieving method (Agrios, 1978; Barker,1985) was used, with slight modifications, for the isolation of the nematode. About 795 g of soil sample was placed in a bucket containing two litres of water. Contents of the bucket were stirred for 20 seconds and left to settle for 60 seconds. The supernatant was then decanted onto a 1.4 mm sieve held over an empty bucket. The water in this bucket was poured over a 0.036 mm sieve held, at a slightly tilted angle, over a third bucket. Nematodes caught on the fine sieve (0.036 mm) were then washed into a petridish.

The nematode c. f. *Protorhabiditis* was maintained on NGM agar plates supporting the growth of *Escherichia coli* (Ward, 1988). NGM had the following components:

peptone	1.25 g
NaCl	1.50 g
agar	8.50 g

0.5g cholesterin/

100ml EtOH	0.50 ml
0.4M KH_2PO_4/	
0.1M K_2HPO_4 pH 6	12.50 ml
1M $CaCl_2$	1.00 ml
1M $MgSO_4$	1.00 ml
distilled water	250.00 ml

E. coli culture was prepared in a broth medium copmposed of:

tryptone	2.0 g
NaCl	0.5 g
glucose	2.0 g
distilled water	300.0 ml

The broth was inoculated with a 24 hour old *E. coli* cultrure and incubated in a water bath at 37°C for 12 hours. The resulting culture was kept at 4°C until used.

Both NGM and the broth medium were sterilised at 121°C for 15 minutes

Before nematodes were transferred to a new NGM agar plate, 0.1 ml of *E. coli* suspension was streaked on the central part of the plate and left overnight in an incubator at 27°C. Then, 0.1-0.3 ml of nematode suspension was placed on the plate. The nematodes were left to grow at room temperature.

The *E. coli* strain used for this work is maintained on nutrient agar slants, at the department of Biology, Addis Ababa University.

3.2. Isolation and maintenance of nematophagous fungi

Drechsler's soil sprinkle technique (Stirling,1991) was applied to soil samples collected from five sites (Betele, Batu Degaga, Kuriftoo, Kurucha, Addis Ababa), of which were tomato farms in areas previously reported to have high incidences of root-knot nematodes (O'Bannon,1975).

About 1 g of each of the soil samples was sprinkled on water agar plates, each seeded with 1000-2000 individuals of c . f. *Protorhabiditis* sp. The plates were observed under binocular light microscope weekly for six to eight weeks. Eight replicate plates were used for each soil sample.

Fungi growing on the sprinkled plates were then transferred to a water agar nematodes (WAN) medium by cutting a block of agar containing fungal mycelia and placing it on WAN. Pure cultures were obtained after a series of transfers on WAN plates.

Pure cultures of the isolated fungi were maintained on Medium A (MA), casein mineral salts medium 1 (CMS-1) or oatmeal agar (OA) slants.

3.3. Growth media

The following media were used for the maintenance of and growth studies on the isolates.

3.3.1. Water agar nematodes (WAN)

agar	2.0 g
distilled water	100.0 ml

After the water-agar mixture has been sterilised, about 15 ml was poured on each of 9 cm petridishes. To each one of these 5 ml of sterilised nematode suspension containing ca 500 individuals of c. f. *Protorhabiditis* sp/ml of distilled water was added.

3.3.2. Medium A (MA)

malt extract	10.0 g
yeast extract	4.0 g
glucose	4.0 g
tap water	1.0 litre

For plates or slants 20 g agar/ litre was added.

3.3.3. Casein mineral salts 1 (CMS-1)

This is a modified form of Blackburn and Hayes synthetic nematode-trapping medium described in Monson *et al.* (1973).

casein	10.00 g
$(NH_4)_2SO_4$	0.02 g
$NaNO_3$	2.00 g
KH_2PO_4	1.00 g

$MgSO_4.7H_2O$	0.50 g
KCl	0.50 g
$FeSO_4.7H_2O$	0.02 g
distilled water	1.00 litre

For plates or slants, 20 g agar/litre was added.

3.3.4. Casein mineral salts 2 (CMS-2)

This medium is different from CMS-1 in only two respects: 20 g casein was added instead of 10 g and ammonium sulphate was not added

3.3.5. Oatmeal agar (OA)

oatmeal extract	1.0 litre
agar	30.0 g

The oatmeal extract was prepared by boiling 30 g of oat flakes bought from local market in 1 litre of tap water and filtering it through a gauze.

3.3.6. Peptone glucose (SPG)

This medium was used by Stadler *et al.* (1993a) for the growth of nematode-trapping fungi.

glucose	4.0 g
peptone	1.0 g
distilled water	1.0 litre

All media components were sterilised at 121°C for 15 minutes.

3.4. *In vitro* efficacy evaluation

A modification of the microwell plate assay method of Stadler *et al.* (1993a) was used in this experiment.

Individuals of c. f. *Protorhabiditis* sp were collected in sterile water from seven to eigh day old NGM agar culture. Excess bacteria were removed by centrifugation (3000 rpm, 10 minutes). The nematodes were resuspended in fresh sterile water.

The assays were carried out in microwell plates with ca. 100 nematodes/ml of sterile water in a well. The nematodes were exposed to four conidial dose levels obtained by serial dilution, a dose being defined as the number of conidia in 1 ml of sterile water applied to nematodes in a well. For each dilution the number of conidia was determined by placing 50 μl of conidial suspension on a cavity slide, counting the conidia under light miroscope (100x magnification), and extrapolating the count to 1 ml. The control well had ca 100 nematodes in 2 ml of sterile water.

Conidial suspensions of the isolates were prepared as follows. The fungi were grown on CMS-1 plates for seven to eight days. The plates were then flooded with 10-15 ml sterile water. To ensure mycelial detachment from the agar surface, the colonies were scraped with a

sterile spatula. The resulting suspension was collected in a sterile flask. Mycelia were separated from conidia by suction filtration through sterile cotton.

Mortality of the nematodes was recoded 72 hours after exposure. The number of dead nematodes was determined by counting motile individuals and subtracting this from the original number of nematodes added before the start of the assay.

For each fungal isolate the assay was repeated three times. Mean mortality data for each of the dose levels were entered to the computer programme Finney's Probit Analysis for Quantal Data which calculated LD_{50} (the lethal dosage that kills 50% of the population) values at 95% confidence limits. Heterogeneity was tested using X^2 values. In each of the three assays conducted it was checked that there was not a more than 5% control mortality.

3.5. Characterization

OA cultures that were seven to14 days old were used to study the distinctive morphological features of the isolates. Identification of the isolates to the genus level was made by reference to Domsch *et al.* (1980). Conidial size was measured under light microscope fitted with a micrometer.

In addition, WAN and CMS-1 were used to observe morphological features that were not apparent on OA.

3.6. Growth optimization

3.6.1. Solid media

A. Growth rate

Three media (MA, OA, CMS-1) were compared for their effects on the growth rates of the fungal isolates. Circular agar pieces, 9 mm in diameter, supporting growth of isolate mycelia were transferred from seven day old MA/OA cultures to the media. The cultures were incubated for six days at room temperature. Colony diameter was measured on the sixth day. Means of three separate experiments for each isolate and medium were used for comparisons.

B. Spore yield

The effects of the above three media on conidial production of the isolates were compared. Conidia were harvested using the procedure described in 3.4., in 30 ml sterile water. From this suspension, after it has been vigorously shaken, 1ml was taken, and diluted 20X (1:20). Conidial count was made as described in 3.4. The means of five separate experiments were used for comparisons.

C. Effect of temperature

The effect of temperature on the growth rates of the isolates was studied by growing the fungi on MA for four days at room temperature (R), 28°C, 32°C, and 37°C. Measurement of colony diameter was taken on the fourth day. Means of three replicates were used for comparison.

3.6.2. Submerged cultures

A. Medium effect on biomass

Three broth media (MA, SPG, CMS-1) were compared for their effects on the biomass yield of the isolates. Agar blocks of 4 cm^2 from seven day old MA/OA isolate cultures were used as inocula. The isolates were cultivated in 250 ml Erlenmeyer flasks, each containing 100 ml of MA, SPG or CMS-1, on a rotary shaker (120 rpm) at room temperature. After eight days of growth, the mycelia were collected on pre-weighed filter papers by suction filtration (Campbell *et al.*, 1978). The papers (and mycelia) were oven dried at 50°C to constant weight. Mycelial dry weight was determined by subtracting the original weight of the filter paper from the combined weight of mycelia and filter paper. The biomass yield was expressed as the mycelial dry weight per 100 ml of broth medium (mg/100 ml). The means of three separate experiments were used for comparisons.

B. pH effect on biomass

The variations in biomass yield of the isolates due to pH variations of MA were investigated in the pH range of 4-10. The inoculation as well as biomass measurement procedures were as described in 3.6.2.a.

3.7. Protease production

3.7.1. Production test

Preliminary tests for protease production were conducted by growing the isolates on nutrient agar gelatin medium (Hankin and Anagnostakis, 1975). The medium was prepared by adding

5 ml of 8% gelatin solution to 100 ml of nutrient agar medium immediately after both have been separately sterilised.

After incubating the cultures at room temperature for three to fivedays, the plates were flooded with an aqueous saturated solution of ammonium sulphate. Formation of a clearing zone around a colony, due to precipitation reaction, was used as an indication of protease production.

3.7.2. Production in submerged culture

Agar blocks of $4cm^2$ from seven day old MA cultures were used to inoculate one litre Erlenmeyer flasks containing 200 ml of CMS-2. The cultures were incubated at room temperature on a rotary shaker (120 rpm) for six to nine days. Culture filtrates, obtained by suction filtration through Whatman No 1 filter paper, were collected on the sixth, seventh, and ninth days of incubation and stored at -20°C until used.

3.8. Protease concentration

Crude enzyme extract, 200 ml in volume, obtained as desrcibed in 3.7.2, was precipitated with ammonium sulphate that gave a 70% saturated solution. It was then cetrifuged at 5000 rpm for 15 minutes. After decanting the supernatant the precipitate was dialyzed against 4 litres of distilled water for one hour. It was further dialyzed against four litres of 50 mM Tris-HCl buffer, pH 7.76 for one hour. The resulting concentrate was stored at -20°C until used.

3.9. Protease activity

Protease activity in the crude and concentrated extracts was measured by using casein as a substrate. To 1 ml of casein suspension (0.605g/100 ml of 50 mM Tris-HCl buffer, pH 7.76) 1 ml of enzyme extract was added. Assay tubes to which 1 ml casein suspension and 1 ml buffer were added were used as blanks. Assay mixtures were incubated at a required temperature for one hour in a water bath. All assays were carried out in duplicates. Immediately after one hour, 2 ml of 10% trichloroacetic acid (TCA) was added to all assay tubes to stop further reaction. Enzyme blanks were prepared by adding 1 ml enzyme extract to an incubated assay tube cotaining 1ml of casein suspension and 2 ml of 10% TCA. The samples were then centrifuged (5000 rpm, 10 minutes). To 500 μl of the supernatant of a centrifuged sample 2.5 ml of 0.5 M Na_2CO_3 and 500 μl of 1:10 diluted phenol reagent were added. This was followed by vortexing the mixture. After 30 minutes at room temperature, the A_{660} of the peptides released in the supernatant was measured using a Bausch and Lomb Spectronic 21 spectrophotometer.

3.10. Protease temperature profile

To determine the optimum temperature at which protease of an isolate shows the highest activity, assays were carried out using enzyme concentrates in the temperature range of 30-70°C at intervals of 5°C. The assay procedure was as described in 3.9. Measured absorbance values were then converted to relative activity (%) by dividing a given absorbance reading by the highest value and multiplying the ratio by 100.

3.11. Protease pH profile

The effects of pH on protease activity were investigated by preparing casein suspensions in the range of of 6-12, followed by assays, using enzyme concentrates. Three buffers were used: phoshate, for the range of 6-8; Tris-HCl, for the range of 7.5-8.5; and glycine-NaOH, for the range of 9-12. Assay procedure and conversion of absorbance values in to relative activity were as described in 3.10.

3.12. Quantification of protease production

To determine whether there was a difference between isolates in the amount of enzyme produced, crude enzyme extracts from nine day old cultures were subjected to assay procedure. Absorbance readings were then converted to concentration values by extrapolation on a standard curve constructed for known concentrations of the amino acid tyrosine. Enzyme production was expressed in units/ml/hr (U/ml/hr), 1 unit being defined as the amount of enzyme that releases 1 μg of amino acid equivalent to tyrosine per hour under the assay condition

The tyrosine standard curve was drawn as follows. From a stock solution of tyrosine (0.02g/100 ml of 50 mM NaOH, pH 10) giving a concentration of 200 μg/ml, serial dilutions that gave 5, 10, 20, 40, 60, 80, 100, and 140 μg tyrosine/ml of the buffer were made. To each 500 μl of these, 2.5 ml of 0.5 M Na_2CO_3 and 500 μl of 1:10 diluted phenol reagent were added. This was followed by absorbance measurement at 660 nm. A plot of tyrosine concentration (μg/ml) versus absorbance, followed by drawing of a regression line was made on a log paper.

3.13. Protease effect on nematodes

Serial dilutions (undiluted or 1X, 2X, 4X, 8X, 16X) of enzyme concentrates from nine day old cultures were used for assays on nematodes. The assay procedure was different from that described in 3.9 in only one respect: a suspension of live *Protorhabiditis* sp. individuals in 50 mM Tris-HCl, pH 7.76 buffer was used as a substrate. Ca 600 nematodes/ml of the buffer were added to each assay tube. Absorbance readings were converted to tyrosine equivalents (μg/ml) by extrapolation on the standard curve used in 3.12. Enzyme blanks were prepared for each dilution.

3.14. Greenhouse trial

Three week old seedlings of the nematode susceptible tomato cultivar Rutgers were transfered to plastic bags each containing 500 g of sterilised reddish sandy soil with a pH value of 5.41. Each plastic bag in a set of 10 bags was inoculated with 0.45 g of chopped *M. incognita* infected tomato root and a specified conidial dose of a fungal isolate. A second set of 10 was inoculated with only 0.45 g of the infected tomato root per bag. Bags of the third set received neither nematode infected root nor isolate conidia. All seedling transplants were made 24 hours after treatments. To avoid nematode cross-contamination between pots, each pot was placed in a paper pot filled with sand 10 cm short of the brim. The pots were arranged in a completely randomized design (CRD).

The seedlings were left to grow for a period of six weeks at the end of which they were uprooted. The effects of the treatments on the growth of the seedlings were compared by

measuring shoot length, shoot biomass, and root length. Biomass was determined by drying shoots to constant weight at 50°C in oven and taking dry weight measurements.

Mean dry weight values were used for comparisons. Significance of the difference between means of treatment groups was determined using t-test (Aggarwal, 1990). Percent yield loss (YL) was calculated using the formula:

$$YL = (1 - X_{SB}/X_{SB0+0}) \times 100$$

where, X_{SB} = mean shoot biomass tomatoes of any treatment group and

X_{SB0+0} = mean shoot biomass of tomatoes neither infected with nematodes nor treated with the fungal isolates.

4. RESULTS

4.1. Identification and characterization of fungal isolates

Two nematode-trapping (predaceous) fungi, coded ART-1 and ART-2, were isolated from soil samples collected from the Addis Ababa University Science campus and Kuriftoo (near Wonji), respectively. Both the isolates were found to belong to the genus *Arthrobotrys*.

Some of the characteristic morphological features of the isolates on OA are listed in Table 3. ART-2 grows faster and has greater number of conidia than ART-1. Conidia of ART-1 are larger in size than those of ART-2. ART-1 has pink colony colour while colony colour in ART-2 range ofs from mostly hyaline (colourless) to slightly pink. ART-1 conidia are arrange ofd in a cluster on conidiophore tips while those of ART-2 form several clusters along a conidiophore (from base to tip). Both isolates produce simple to three-dimensional traps (adhesive networks or adhesive nets) when grown on WAN and CMS-1. Trap formation was best observed in the presence of nematodes (Fig. 2).

4.2. Efficacy evaluation

In vitro comparison of the nematode-destroying capabilities of the isolates showed that ART-1 was more efficient than ART-2 in destroying nematodes (Table 4). The LD_{50} value for ART-1 was found to be two and half times lower than that of ART-2.

4.3. Growth optimization

4.3.1. Solid media

4.3.1.A. Growth rate

Out of the three media (MA,OA,CMS-1) that were compared, OA gave the greatest lateral growth (mean colony diameters of 6.3 cm and 7.8 cm for ART-1 and ART-2 respectively) while CMS-1 gave the least (mean colony diameters of 3.6 cm and 5.6 cm for ART-1 and ART-2 respectively). ART-2 grew faster than ART-1 on all the media (Fig. 3).

4.3.1.B. Spore yield

As can be seen in Fig. 4, there is a remarkable spore yield difference between the isolates. ART-1 had much lesser spore yields than ART-2 on all the media investigated: 4080 conidia/ml on MA, 4960 conidia/ml on OA and 1040 conidia/ml on CMS-1. Even though ART-2 gave its smallest spore yield when grown on CMS-1, the spore count value was more than two fold the maximum spore yield of ART-1: 14560 conidia/ml. It gave higher spore yields on MA and OA: 28240 and 31400 conidia/ml, respectively. OA was found to be optimal for the production of spores for both isolates.

4.3.1.C. Effect of temperature

Both isolates attained greatest colony diameter when incubated at 28°C: 5 cm for ART-1 and 5.7 cm for ART-2 (Fig. 5). Incubation at 37°C resulted in little lateral growth: colony diameter of 1 cm was observed for both isolates. However, best mycelial biomass and sporulation were observed on visual inspection when the fungi were incubated at room temperature.

4.3.2. Submerged cultures

4.3.2.A. Medium effect on biomass

MA gave the highest biomass yield for both isolates: 631 mg/100 ml and 755 mg/100 ml for ART-1 and ART-2, respectively. Though ART-2 gave greater yield than ART-1 (Fig. 6), there was little difference between the isolates when grown in SPG. However, ART-1 which was found to be a slow grower on all solid media tested gave a higher yield than ART-2 when grown in CMS-1: 363 mg/100 ml as compared to 278 mg/100 ml for ART-2 (Fig. 6).

4.3.2.B. pH effect on biomass

As can be seen in Fig. 7 that there is an appreciable difference in biomass yield between the isolates in the pH range of of 4-5: 436-478 mg/100 ml for ART-1 and 509-669 mg/100 ml for ART-2. ART-2 grew well in the acidic range of. ART-2 grew more or less constantly in the range of of 5-9, and gave minimal biomass yields at the extreme values of 4 and 10. ART-1 gave an optimal yield in the range of 7-8 of: 487-535 mg/100 ml.

4.4. Protease production

4.4.1. Production test

Preliminary tests on solid medium showed that both isolates had proteolytic activity. This led to the production in and collection of proteases from submerged CMS-2 cultures.

4.5. Protease temperature and pH profiles

Proteases from both of the isolates were found to have a temperature optimum of 50°C (Fig. 8). Concentrates from six day old cultures were used for this experiment.

The pH optima of the proteases from ART-1 and ART-2, in assays conducted at 50°C, were determined to be in the range ofs of 7.5-8 and 7.5-9, respectively (Fig. 9). Enzyme concentrates from six day old cultures were used for the assays.

4.6. Quantifiacation of protease production

Assays conducted at 50°C using crude enzyme extracts from nine day old cultures showed that ART-1 produces more protease than ART-2: 19 U/ml/hr and 14 U/ml/hr, respectively.

4.7. Protease effect on nematodes

A comparison of various dilutions of proteases from the isolates has shown that proteases from ART-1 were found to be more efficient in degrading nematodes than those from ART-2 (Fig. 10).The amount of amino acids released after the nematodes have been degraded was expressed in terms of equivalents of the amino acid tyrosine (μg/ml). For each dilution factor, the amount of amino acids released by proteases from ART-1 was greater than the amount released by ART-2 proteases. Assays for this particular experiment were carried out at 32°C to avoid nematode disintegration due to heat. Enzyme concentrates from nine day old cultures were used in the assays.

4.8. Greenhouse trial

About 60000 conidia of ART-1 were added to each *M. incognita* infected pot. ART-2 was added at a dose level of 180000 conidia per nematode infected pot. Preliminary experiments were conducted in the greenhouse to decide upon the conidial dose level for each isolate. Mean shoot length (SL), root length (RL), and shoot biomass (SB) values of nematode infected tomatoes, treated with either one of the fungal isolates, were significantly greater than those nematode infected tomatoes that have been treated with neither one of the isolates (tables

5 and 6). Tomatoes grown in pots to which neither *M. incognita* nor the fungi were added had significantly greater mean SL, RL, and SB values than any other treatment group. Percent yield loss (YL) was highest in tomatoes treated with only *M. incognita* (69.7%). Nematode infected tomatoes treated with either of the fungal isolates, on the other hand, had lower losses: 44.5% for ART-1 treated and 44% for ART-2 treated tomatoes. ART-2 required 3X the inoculum density of ART-1 (180000 conidia/pot) to achieve a yield loss lowering effect similar to ART-1.

DISCUSSION

Predaceous fungi producing adhesive nets are usually thought of as having rapid growth in cultures, as having more of a saprophytic existence in soil, and as being relatively inefficient predators (Domsch *et al.*, 1980; Sayre and Walker, 1991; Stirling, 1991). These generalizations often tend to ignore the variations in nutritional utilization that exists among net-forming species. Both ART-1 and ART-2 are net-forming species (Fig. 2). And yet there is a significant difference, since the 95% confidence intervals do not overlap, in predaceous efficiency between them.

Results of growth investigations show that ART-1 is relatively a slow growing isolate. During earlier stages of this work, establishing pure cultures for ART-1 proved to be more difficult than for ART-2. Thus, ART-1, in relative terms, appears to be less saprophytic and more nematode dependent than ART-2.

Although both isolates attained greatest colony diameter at 28°C, best mycelial biomass and sporulation were observed at room temperature. Optimal temperature for the lateral growth of *Arthtobotrys conoides* is known to be in the range of 20-28°C; *A. oligospora* grows well at 20°C; and *A. superba* requires an optimum range of of 20-22°C (Domsch *et al.*, 1980). The highly restricted growth of the fungi at 37°C is in agreement with previous observations that *Arthrobotrys* spp. do not grow well at temperatures above 35°C (Domsch *et al.*, 1980; Nordbring-Hertz *et al.*, 1995).

In two of the broth media used, ART-1 gave lower biomass yield than ART-2, which was in agreement with the observation that ART-1 is a slow grower. However, the situation was

reversed when the isolates were grown in CMS-1 broth medium: ART-1 gave greater biomass than ART-2. In an attempt to explain this discrepancy, the following hypothesis was formulated and tested: ART-1 produces proteases in larger quantity than ART-2, and is thus in a better position to utilize the casein substrate and convert it to a larger biomass. Results of the assays carried out to quantify protease production by the isolates support this hypothesis.

The significance of protease production in host-pathogen interaction is well noted (St. Leger *et al.*, 1987a,b). Many attempts have been made to establish a direct correlation between a pathogen's efficiency in host colonization and protease activity (St. Leger *et al.*, 1988; Charnley, 1991). To test this line of argument, proteases from the 2 isolates were compared with respect to their efficiency in degrading nematode cuticle. The results revealed that proteases from ART-1 were more efficient (Fig. 10). Because ART-1 proteases are produced in greater quantity than those of ART-2, for every dilution on the plot their activity was found to be higher. The nematode cuticle provides a protective barrier against fungal penetration. The sooner it is removed , the higher the chances of fungal invasion. ART-1 seems to ensure this by producing proteases at high levels. Work with the locust pathogen *Metarhizium anisopliae* has led to the identification of a specific protease as a pathogenicity determinant (St. Leger et al., 1988). The fungus is known to produce this protease, called PR-1, in a large quantity.

Of the three solid media tested for enhancing lateral growth rate and spore yield, OA was found to be suitable. Although the isolates grew faster on this medium, it was observed that there was a very sparse mycelial growth. Best mycelial growth of the isolates was observed on MA. This was the reason why OA was excluded from and MA was chosen for investigations on biomass yield and effects of pH in submerged cultures instead. The optimal

biomass yield of the isolates in MA broth is attributed to the yeast extract component of the medium. It is known that zinc ions and the vitamines biotin and thiamine are required for mycelial growth of *Arthrobotrys* spp. (Nordbring-Hertz, 1973; Domsch *et al.*, 1980). These vitamines and ions are essential constituents of yeast extract.

Though both of the isolates had a more or less restricted growth on solid as well as in broth CMS-1 medium, it was found out that (aside from WAN) it was on this medium that they retained their nematode-trapping habit: it induced trap formation in the isolates. The casein component of the medium was responsible for bringing about this morphogenic change in the isolates. Nordbring-Hertz (1973 and 1974) has shown that casein hydrolysis products are capable of inducing such morphogenesis in *A. oligospora*.

The investigations on the pH requirements for growth indicated that there was little difference in biomass yield between the isolates in the pH range of 6-9. It was only in the range of 4-5 that a marked difference was observed (Fig. 7). ART-2 grew best in the acidic range of. This has got an important ecological implication: ART-2 can have increased survival and better performance than ART-1 in acidic soils. Previously reported optimal pH values for the growth of *Arthrobotrys* spp. include: 4-6 for *A. conoides*, and 5-7 for *A. oligospora* (Domsch *et al.*, 1980). Norton (1978) has pointed out that there is nothing "unique about the pH requrements" of nematode-trapping fungi until "extreme ranges of are attained." For instance, *A. conoides* and *A. oligospora* had little or no growth at pH values of 9.5 and 2.5, respectively (Corton, 1978).

Proteases fom both of the isolates have a temperature optimum of 50°C (Fig. 8). This value is greater than that reported for proteases from *A. oligospora* (37°C) (Tunlid and Jansson, 1991)

and less than that reported for protease from the nematode egg-parasite *P. lilacinus* (60°C) (Bonants *et al.*, 1995). A possible ecological implication of this is that the isolates can have enhanced nematode invasion capability in soils found in warmer ecological zones.

The pH optimal values, 7.5-8.1 for ART-1 and 7.5-9 for ART-2, of the proteases are more or less in agreement with previously reported ones: 7.5-9 for *A. oligospora* (Tunlid and Jansson,1991) and 10.3 for *P. lilacinus* (Bonants *et al.*, 1995). Since the major constituent of the nematode cuticle is collagen (Pechenik, 1985), nematophagous fungi produce collagenases during penetration of nematodes (Schenk *et al.*, 1980; Tunlid and Jansson, 1991; Bonants *et al.*, 1995). So far collagenases from nematophanous fungi are known to be serine proteases. Almost all serine proteases are known to be optimally active in the alkaline pH range of (North, 1982).

In the greenhouse trials the differences in mean SL, RL, and SB values between isolate-treated, nematode infected tomatoes and the tomatoes in the nematode infected pots not treated with the fungi indicate that a measure of nematode suppression has been achieved by both ART-1 and ART-2. The yield loss results showed that treating the nematode infected tomatoes with either one of the fungal isolates reduced yield loss by about 25%. ART-2 required three times the inoculum density of ART-1 (180000 conidia per pot) to attain a suppression effect of more or less similar to ART-1. This was in agreement with the *in vitro* observation in which ART-2 was less efficient than ART-1. However, the mean SL, RL, and SB values of tomatoes treated with the fungi were significantly less than those neither infected with nematodes nor treated with the fungi. This suggests that nematode suppression has not been fully achieved by the isolates. The level of root gall formation in all nematode infected tomatoes (whether treated with the fungi or not) appeared, up on visual inspection, more or less similar, suggesting that control has not been fully attained.

In his review of trials conducted to assess nematode suppression capabilities of nematode-trapping fungi, Stirling (1991) has stated that *Arthrobotrys* spp. had , more often than not, little or no effect on soil nematode populations. Nematode-trapping fungi have generally been tested against the cyst and root nematodes which spend the better part of their life cycle inside plant root tissues. The nematode-trapping activity of these fungi often does not coincide with the presence of the juvenile, free living stages of the nematodes. Stirling (1991) considers this the major reason for the lack of consistent and adequate control of root-knot nematodes by these fungi. The results of the greenhouse experiment show that nematode control by ART-1 and ART-2 has to some extent taken place. Since there were no organic amendments added to the soil in which the tomatoes were grown, the observed nematode suppression can fully be attributed to the fungal isolates that were added.

Recent investigations on the nematode-trapping fungus *A. oligospora have suggested that* it is possible to improve the survival and trapping activity of such fungi in soil (Dackman and Nordbring-Hertz, 1992; Nordbring-Hertz *et al.*, 1995). The improvement essentially involves subjecting conidia to mutation or chemical treament so that the conidia form adhesive traps. The traps are regarded as survival structures that enhance the trapping activity of the fungi. Thus, it is also possible to increase the nematode-suppression effects of ART-1 and ART-2 by using such methods.

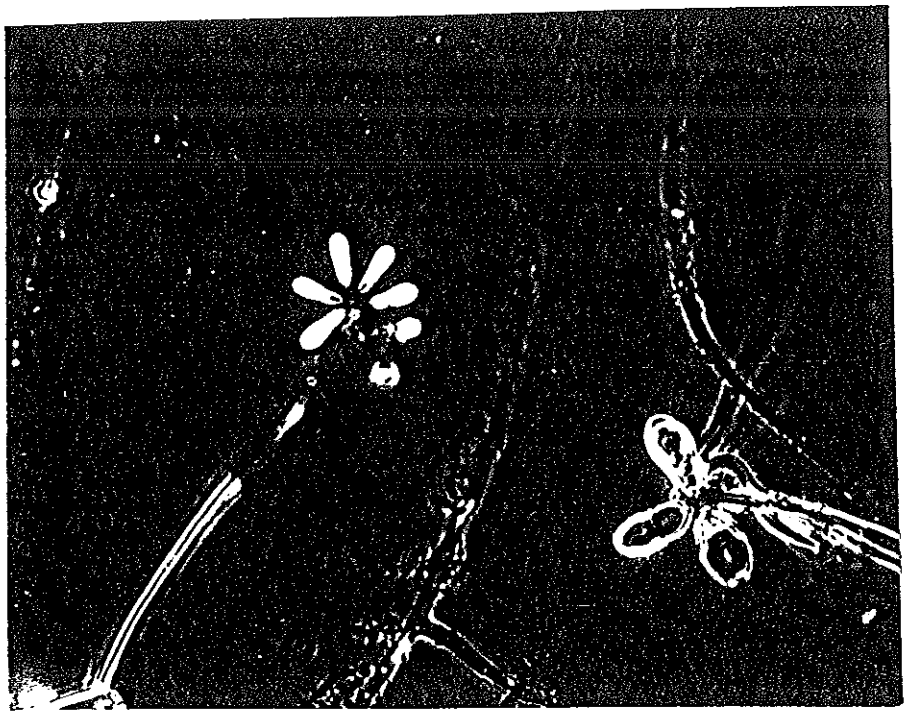
Table 3. Some morphological features of the isolates when grown on OA.

Isolate	Colony colour	Colony diameter (cm)	No. of conidial clusters/conidiophore	Conidial size, length X width (μm)
ART-1	pink	3 in 3 days	1 mostly, 4 rarely	18-34x10-15.75
ART-2	often colourless, seldom slightly pink	4 in 3 days	1-6, 4and5 mostly	15-28x10-17

Table 4. LD_{50} values of the isolates

Isolate	LD_{50} (No of conidia/ml)	95% CI	$X^2_{(2)}$
ART-1	290	229-352	0.7643
ART-2	725	385-905	1.7091

a



0.05mm

b

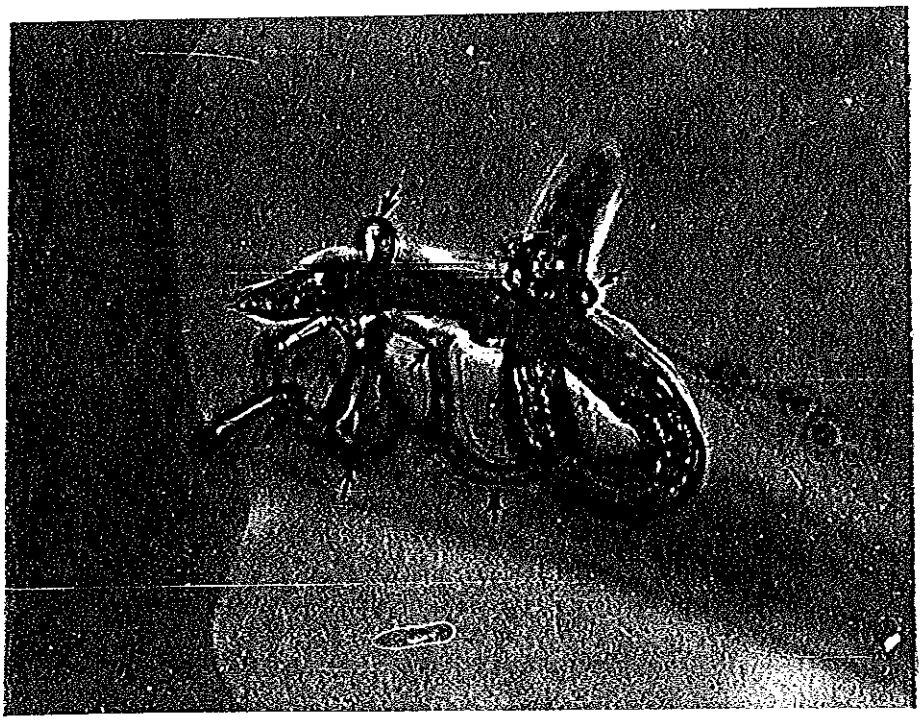
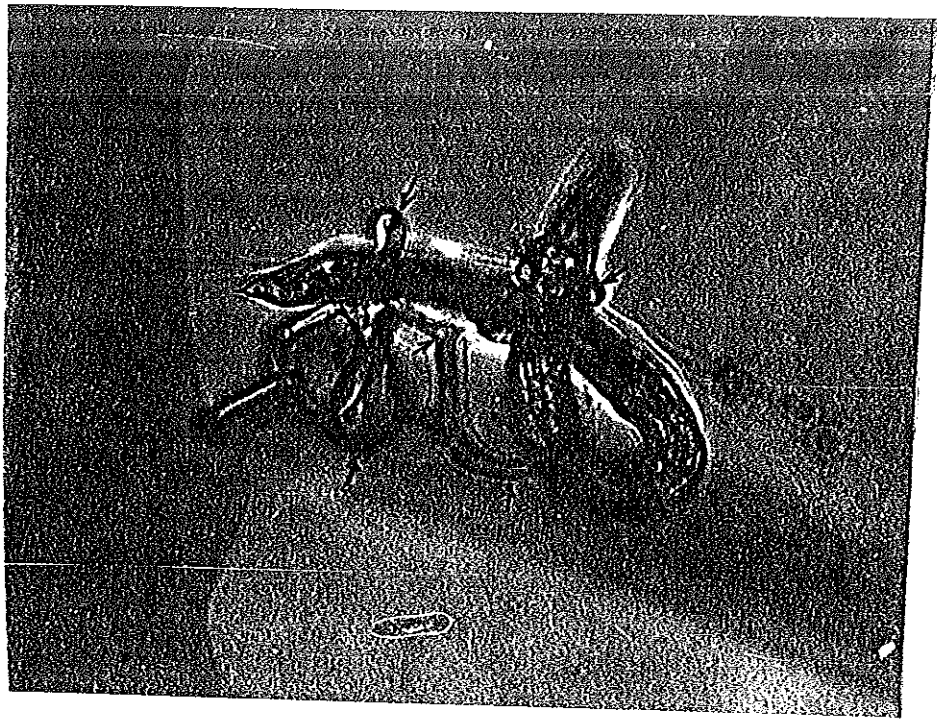


Fig. 2. Sporulation and trap formation by ART-1 and ART-2 when growen in the presence of the nematode c. f. *Protorhabiditis* sp . (a) Sporulation of ART-1. (b) and (c). A nematode caught in an adhesive net of ART-1. (d), (e), (f) Adhesive nets of ART-2 in which a nematode is caught.

c



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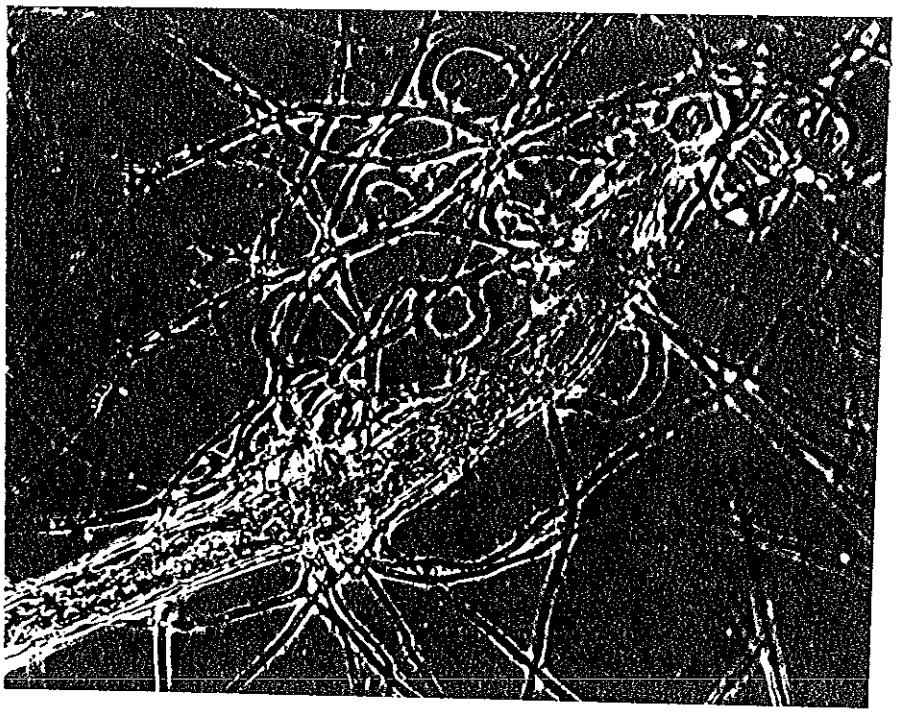

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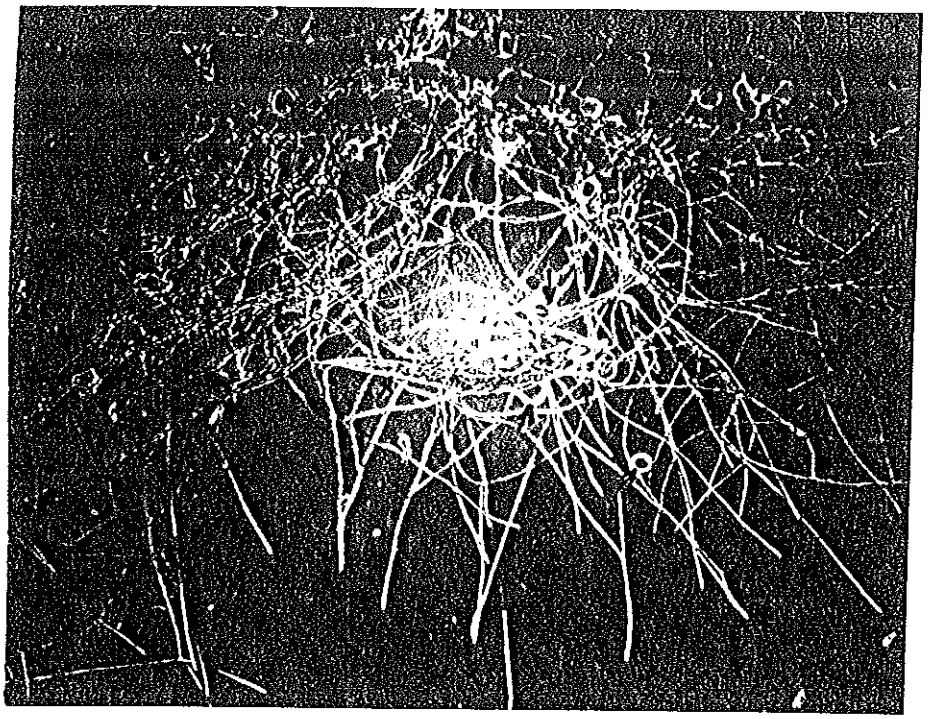

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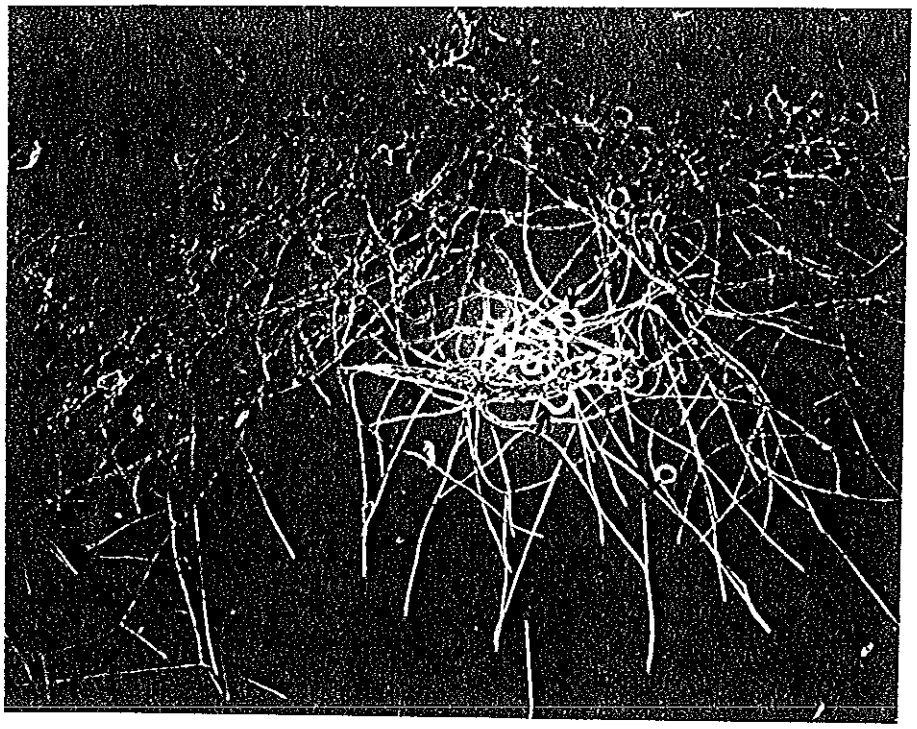

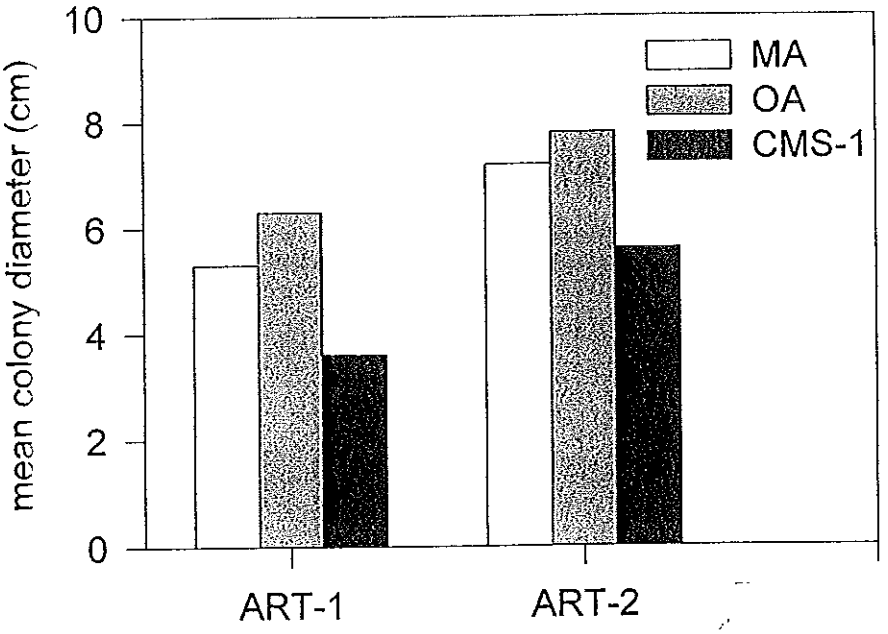

Fig.3 Effect of medium on growth rate

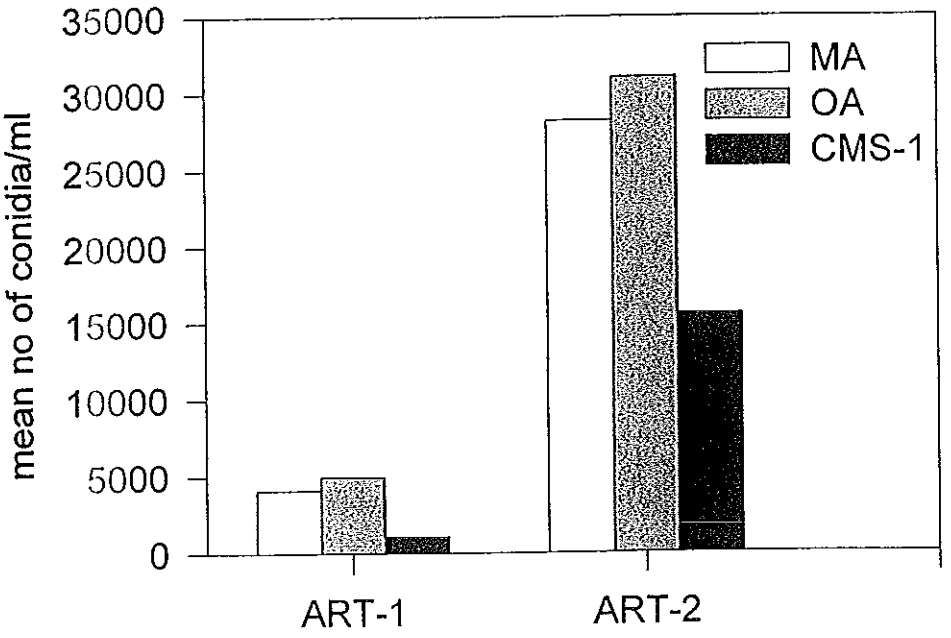

Fig. 4 Effect of growth medium on spore yield.

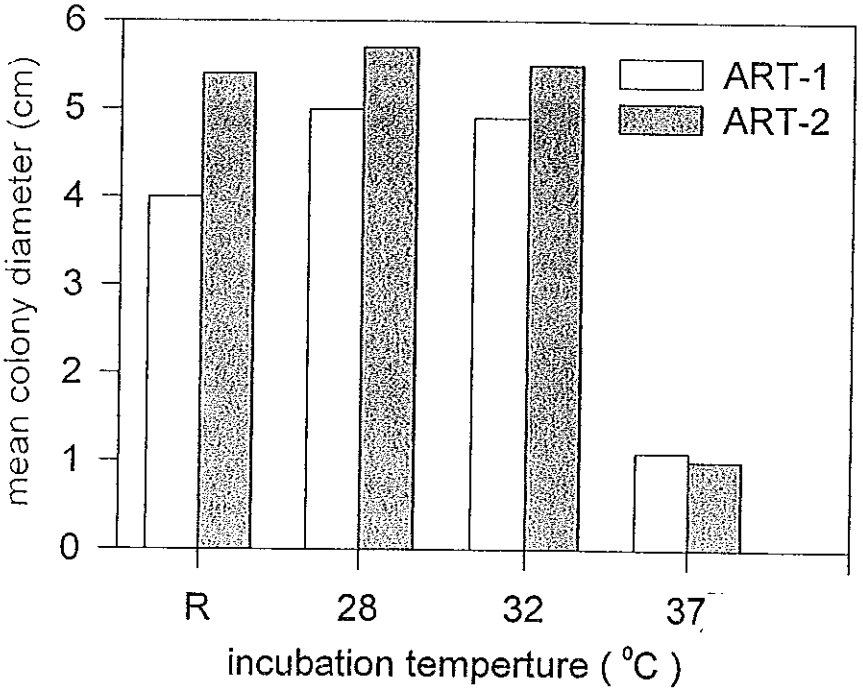

Fig. 5 Effect of incubation temperature on growth rate
R= room temperature. MA
was used as growth substrate.

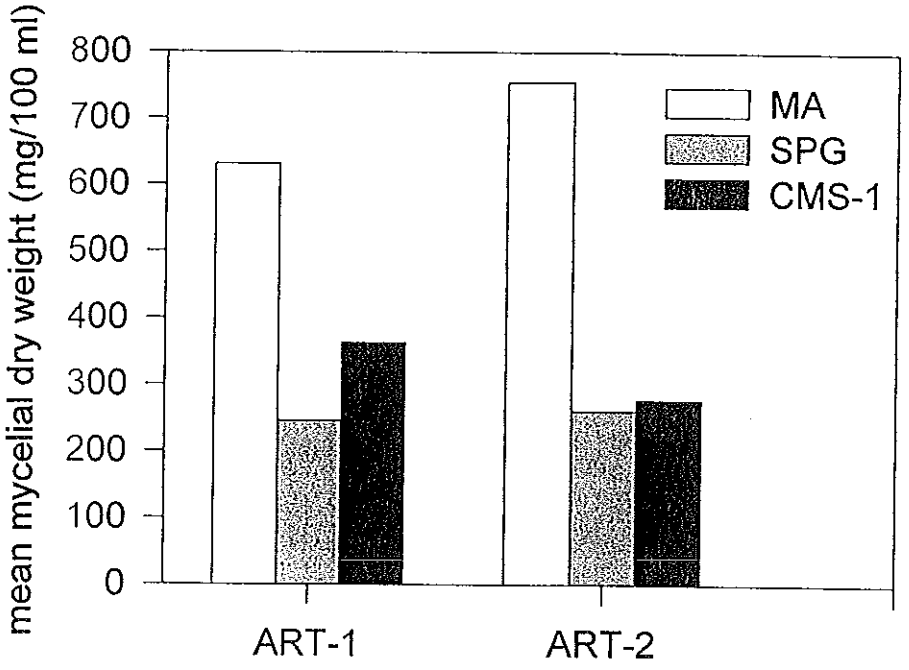

Fig. 6 Effect of medium on biomass yield.

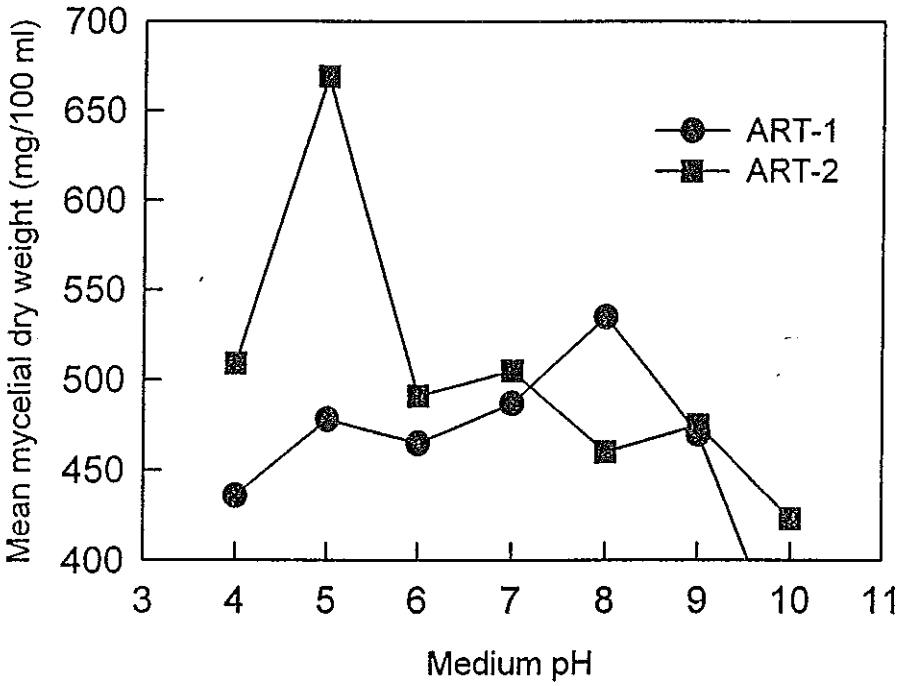

Fig. 7. Effect of medium pH on biomass and yield. Broth of MA was used for growth.

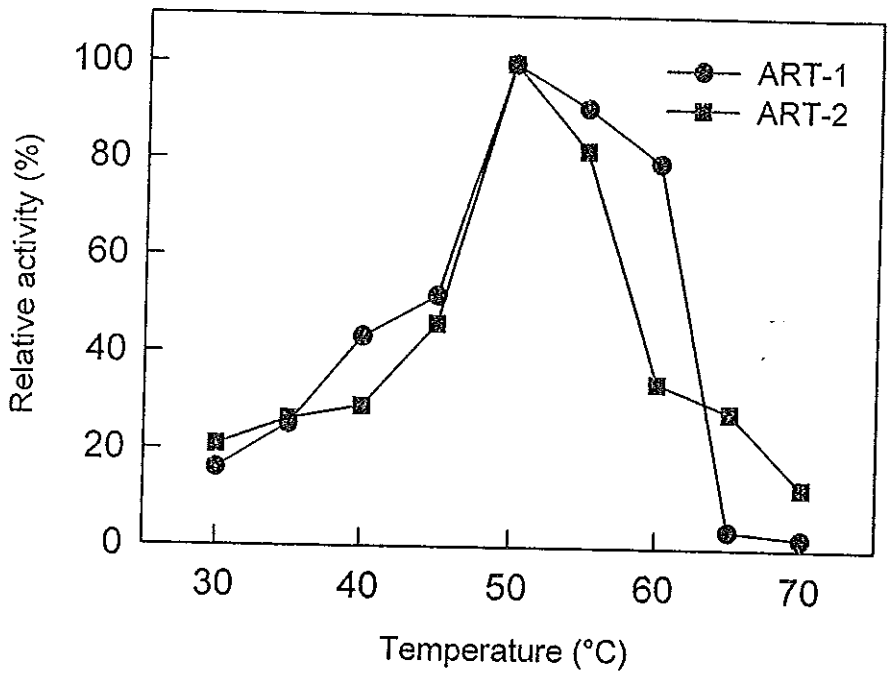

Fig. 8. Temperature profiles of the proteases from the two isolates

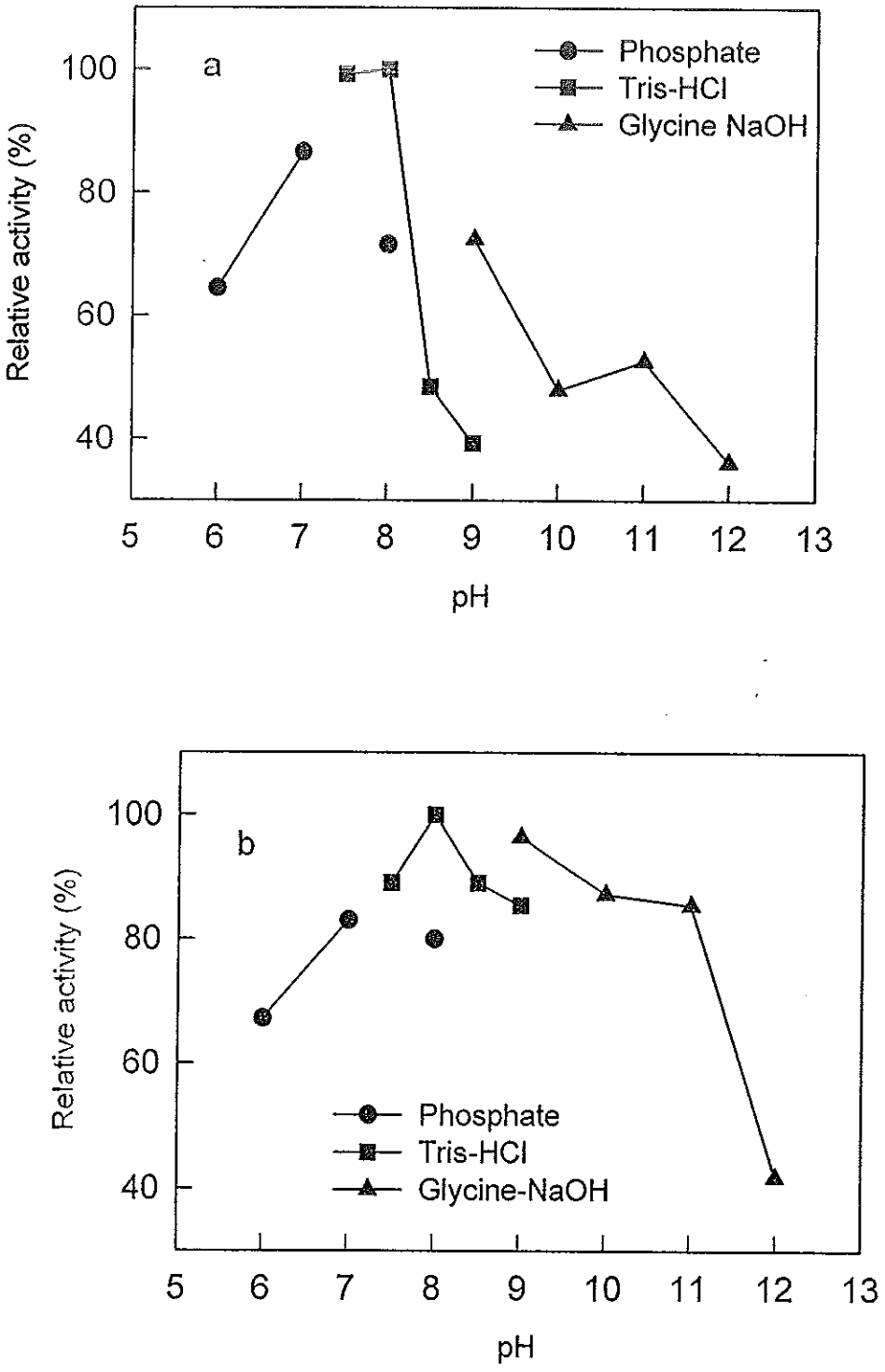

Fig. 9. pH profile of proteases from (a) ART-1 and (b) ART-2.

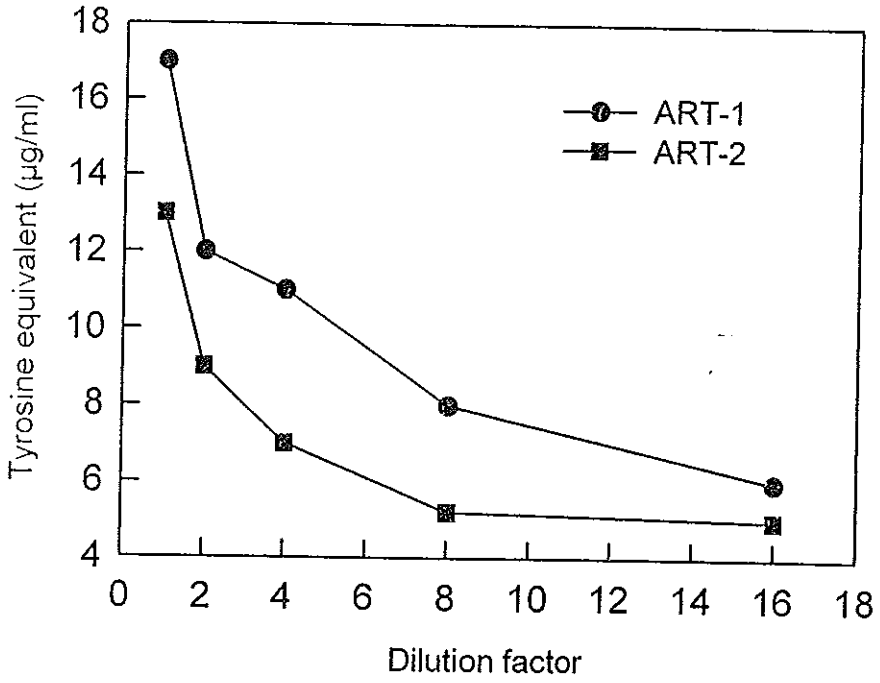

Fig. 10. Nematode-degrading capabilities of different dilutions of proteases from ART-1 and ART-2.

Table 5. Effect of nematodes and the test isolates

on the growth of the tomstoe cultivar Rutgers.

Treatment No	Treatment	SL (cm)	RL (cm)	SB (mg)	YL (%)
1	N+I	13.73	16.9	97	44.5
2	N+II	11.75	18.4	98	44
3	0+0	15.17	22.5	175	0
4	N	9.25	13.7	53	69.7

N+I= nematodes and conidia of ART-1;

N+II= nematodes and conidia of ART-2;

0+0= treated with neither nematodes nor the isolates;

N= nematodes only;

SL= mean shoot length (cm);

RL= mean root length (cm);

SB= shoot biomass (mg).

YL= yield loss (%)

n= 10

Table 6. Calculated t-values between treatment groups.

	a. SL b. RL c. SB	Treatment number 2	 3	 4
	1	a)3.49 b)5.05 c)0.05	a)3.45 b)4.73 c)5.47	a)10.14 b)2.54 c)10.24
TN	2		a)6.27 b)3.16 c)3.42	a)5.62 b)3.51 c)7.38
	3			a)14.2 b)8.19 c)8.72

t-tabulated (degree of freedom=18, p=0.05)= 2.10. TN = Treatment number. If t-tabulated is less than t-calculated between any two treatment groups, then there is a significance difference between their means.

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