PATHOGENIC SOIL FUNGI AND PLANT PARASITIC NEMATODES ASSOCIATED WITH CARROT IN EASTERN HILLS OF NEPAL

DISSERTATION

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PARTIAL FULFILLMENT FOR THE DEGREE OF MASTER OF SCIENCE IN BOTANY

BY

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CERTIFICATE

This is to certify that the dissertation entitled **"Pathogenic Soil Fungi and Plant Parasitic Nematodes Associated with Carrot in Eastern Hills of Nepal"** was carried out by **Mr. Mahesh Prasad Pudasaini** under my supervision. This work has been accomplished on the basis of candidate's original research work and submitted here for the partial fulfillment of the degree of **Master of Science in Botany (Plant Pathology)**. To the best of my knowledge, the results of this work have not been submitted for any other degree. Therefore, I'm pleased to forward this dissertation for the final approval and acceptance.

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LETTER OF APPROVAL

This dissertation entitled **"Pathogenic Soil Fungi and Plant Parasitic Nematodes Associated with Carrot in Eastern Hills of Nepal"** submitted by **Mr. Mahesh Prasad Pudasaini** has been accepted as partial fulfillment for the requirement of **Master of Science in Botany (Plant Pathology)**.

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Mahesh Prasad Pudasaini
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Summary

A taxonomical study and interaction of the pathogenic soil fungi and the parasitic nematodes present in rhizosphere and roots of carrot from Tehrathum and Dhankuta districts of Eastern Nepal were undertaken. Samples were collected from two locations Tehrathum district and three locations of Dhankuta district. Fungi were isolated from both soil and root samples using general and selective media required for most pathogenic soil fungi. Different colonies were finally purified and cultured on selective media in order to identify. In order to collect morphometrical data and morphological features, fungi were cultured on different media required for such (given in appendixes). Nematodes were extracted from both soil and plant samples using extraction machines or simple Bearmann funnels (Seinhorst, 1950; Pudasaini *et al.*, 2006).

Both fungi and nematodes mounted on slides were observed under light microscope (Wild Heerbrugg, M12, Swiss made). All drawings were made in 1000x magnification to get better view. Morphometrics were taken using an Olympus BX50 compound microscope equipped with Leica image-capture IM500 system and software.

On the basis of morphometrics and morphological characters, nine fungi species and six nematodes species have been identified. The fungal species were *Pythium aphanidermatum, P. ultimum, P. irregulare, P. sulcatum, Fusarium oxysporum, F. solani, F. moniliformae, Rhizoctonia solani* and *Scleritinia sclerotiorum*. The nematode species were *Pratylenchus penetrans, P. neglectus, Meloidogyne hapla, M. incognita, Helicotylenchus dihystera* and *Rotylenchus uniformis.* Among them, the nematode species *Pratylenchus penetrans, P. neglectus* and *Rotylenchus uniformis* are new records from Nepal.

Among the fungal species *Pythium ultimum, P. irregulare, Fusarium oxysporum, F. solani, Rhizoctonia solani, Scleritinia sclerotiorum* were found in all samples collected from all five locations. Among the nematode species only *Meloidogyne hapla* were found in all samples. *Pratylenchus penetrans* were found in all samples except Jeetpur (Dhankuta district). The distribution pattern of *Rotylenchus uniformis* was narrow and found in few sample only.

The interaction between most of the fungi species and nematodes were possible, but the study was limited to *Pythium ultimum* and *Pratylenchus penetrans* on disease development in carrot. The study has been carried out in growth chamber. *Pythium ultimum* caused about 11% yield reduction while *Pratylenchus penetrans* caused about 23% of yield reduction in carrot. The combination of both species caused 40-44% yield reduction indicating the existence of synergistic effect on disease development in carrot.

CHAPTER 1

Introduction

Fungi are organism with their assimilative (non-reproductive) structure made up of a much-branched system of slender tubes known as mycelium. Such mycelium usually grows in a radial manner from the point of origin, if conditions are equal about that point. Each branch of this structure may be divided by cross wall called septa or be continuous, depending on the nature of the species. Ordinarily the growth is confined to a rather narrow zone of plane surface and then the mycelium becomes the circular colony of the Petri dish or the fairy ring of the open field.

In reproduction the fungi produce spores as the result of a cell and nuclear fusion (sexual spores). Under cultural conditions, however, such spores are rather exception and the asexual spores such as clyamydospores, conidia, etc. are produced as a result of cell or mycelium fission which is commonly used to identify the species.

Soil borne diseases of ancient and modern crops have always had some impact on growth and productivity. Soil borne plant pathogenic fungi cause root-rot, crown or collar rot, damping off, blight, fruit decay and wilts in field and horticultural crops. The extent of damage is not known precisely, but it is estimated to be at least 4 billion dollars annually in the United States (Utkhede and Gupta, 1996). The expansion of crop diversity in agriculture has required parallel expansion of strategies to minimize soil borne diseases i.e. manage to maintain diseases at an economic level. Disease management first of all requires the knowledge about the pathogen involved. *Alternaria*, *Botrytis*, *Fusarium*, *Phytophthora*, *Pythium*, *Rhizoctonia* and *Sclerotinia* are common genera of soil pathogenic fungi.

Nematodes are bilaterally symmetrical, unsegmented, appendageless and multicellular invertebrates. They are one of the most diverse groups of animals in form, but common in many biological characteristics. They have a body cavity, a complete digestive tract, a complex nervous system, secretory-excretory system, reproductive and musculature systems (Hirschmann, 1971; Poinar, 1983; Bird and Bird, 1991). However, they lack a specialised

respiratory or circulatory system (Bird and Bird, 1991). The body is covered by a cuticle which helps the identification of most genera based on its surface structure (Bird and Bird, 1991; Decraemer and Hunt, 2006) and being transparent it reveals sufficient anatomical features under a stereoscopic microscope (Baker, 1998).

Nematodes are widespread organisms found in all habitats and ecosystems of the biosphere. They occur in soil, decaying organic matters, all forms of plant life and most animals, including domesticated and wild species (Norton, 1978). Depending upon their habitat, nematodes can be classified into plant-parasites, free-living (terrestrial and marine) and animal-parasites (invertebrates and vertebrates).

Plant-parasitic nematodes are microscopic and associated with all forms of plant life in both natural and agricultural ecosystems. In agricultural ecosystems many plant-parasitic nematodes cause significant damage on crops. Estimated overall average annual yield loss of the world's major crops due to damage by plant-parasitic nematodes was 12.3% and monetary losses due to damage on life sustaining crops were estimated at $77 billion annually based on 1984 production figures and prices (Sasser and Freckman, 1987). On a world wide basis, *Meloidogyne*, *Pratylenchus*, *Heterodera*, *Ditylenchus* and *Globodera* were rated the most economically important genera. Plant-parasitic nematodes have developed a specialised parasitic relationship with their host. Depending upon their parasitic habit they are categorised into (i) ectoparasites (e.g. *Tylenchorhynchus, Xiphinema*), (ii) endoparasites and (iii) semi-endoparasites (e.g. *Tylenchulus*, *Rotylenchulu*s). Endoparasitic nematodes are further divided into migratory endoparasites (e.g. *Pratylenchus*, *Radopholus*) and sedentary endoparasites (e.g. *Meloidogyne*, *Globodera*) (Decraemer and Hunt, 2006).

Carrot (*Daucus carrota* L.) is probably native of western Asia or the Near East, but forms are found in the Mediterranean region, southwest Asia, tropical Africa, Australia and North and South America (Reed, 1976). Now it is distributed all over the world from tropics to the temperate zones. It is cultivated for the enlarged fleshy taproot, eaten as a raw vegetable or cooked in many dishes. Eaten sliced, diced, cut up, or shoe-stringed, carrots are used in many mixed vegetable combinations. They are sold in bunches, or canned, frozen, or dehydrated. They may be baked, sautéed, pickled, and glazed, or served in combination with meats, in stews, roasts, soups, meat loaf or curries.

Carrot is reported to tolerate annual precipitation of 3.1 to 41.0 mm, annual temperature of 3.6 to 28.5°C and pH of 4.2 to 8.7 (Duke, 1978; 1979). Basically, it is a cool season crop, with optimum growth at 16-21°C, requiring for best growths long periods of mild weather free of temperature and moisture extremes. Soils should be deep friable, well-drained, and loams and organic soils such as muck or peat have been used, with pH ranging from 6.5-7.8 (Reed, 1976).

Carrot suffers from many fungal, bacterial, nematode and viral diseases. The major fungal pathogens reported are *Aecidium carotinum, Alternaria* spp., *Botrytis cinerea, Centrospora acerina, Cercospora apii, Fusarium* spp., *Phytophthora cactorum, Rhizoctonia* spp., *Sclerotinia* spp., *Pythium* spp. etc (Reed, 1976). Like most root crops, carrot is susceptible to nematodes. The major nematode species parasitic to carrot are: *Meloidogyne chitwoodi* (Santo *et al.*, 1988), *M. hapla*, *Heterodera carotae, Ditylenchus destructor, D. dipsaci, Pratylenchus crenatus*, *P. penetrans* (Potter and Olthof, 1993), *P. thornei* (Castillo *et al.*, 1995), *P. neglectus* (Siddiqi *et al.*, 1973), *P. vulnus, Hemicycliophora typica, Longidorus elongates* (Hooper, 1973).

Many pathogenic soil fungi as well as many plant-parasitic nematodes are inhabited in the same soil environment. Plant-parasitic nematodes commonly interact with soil borne pathogenic fungi (Miller *et al.*, 1963; Oyekan and Mitchell, 1971; Jin *et al.*, 1991; LaMondia, 1999; 2003). Among these fungi, some are week pathogens and some are strong one. The week pathogenic fungi can also cause great damage to the crops if certain nematodes are present in the same habitat. The wounds produced by the nematode stylet (feeding device of plant-parasitic nematodes which pierce and suck plant cell sap) during feeding provide a means of entry for a wide variety of relatively non-specific soil micro-organisms, including root-infecting pathogens that may also contribute to lesion formation and may enhance the disease (Oyekan and Mitchell, 1971; Manzanilla-López *et al.*, 2004). Several reports have already been published about the interaction between soil pathogenic fungi and plant parasitic nematodes in the number of crops (e.g. Jin *et al.*, 1991; Botseas and Rowe, 1994; Saeed *et al.*, 1998; LaMondia, 1999). Disease complexes due to the interaction of plant-parasitic nematodes and soil borne pathogens were reviewed but no mechanism underlying their interaction has been proven yet (Back *et al.*, 2002). These interactions may have no detectable effect on the host plant, the effects may be additive, or they may be synergistic.

Objectives

In most of the reports, it was repeatedly mentioned that either the fungi enhanced the multiplication of plant parasitic nematodes or the nematodes make the plant a favourable host by opening door to the fungal infection. It is also believed that the nematode infestation cause some modification in the secondary metabolites of host which then became more attractive to fungi that are not pathogenic to that host prior to the presence of nematodes. In other cases, nematodes can lower the resistance of plants to other pathogens. The resistance of peas to *Fusarium oxysporum* f. *pisi* broke down when inoculated with *P. penetrans* but not when wounded with the sterile needle; this suggests that the nematode does more than just wounding the host and letting the fungus in (Oyekan and Mitchell, 1971). Wilt incidence increased in tomato when *Verticillium albo-atrum* was in presence of *P. penetrans* (Conroy *et al.*, 1972).

Therefore, it is very important to know the presence of pathogenic soil fungi such as *Pythium*, *Fusarium*, *Rhizoctonia* and *Sclerotinia* and plant parasitic nematodes in the rhizosphere of carrot and their interactive effect on crop yield. The information about their interaction will help to manage the disease and to increase the crop productivity.

Therefore the specific objectives of this study were to:

(i) Identify the pathogenic soil fungal and plant parasitic nematode community in rhizosphere of carrot culture and

(ii) Determine the interactive effect of fungi and nematode on crop yield.

Justification

The presence of plant parasitic nematodes and plant pathogenic soil fungi at the same time and in the same niche may cause synergistic damage on plants. No such study has been done in Nepal which is very informative in decision support for integrated disease management (IDM) system. Therefore, this study was justified and undertaken.

Limitations

There are enumerable soil pathogenic fungi and plant parasitic nematodes associated with many crops throughout the country. In the given limited time, resources and energy, it is not possible to carry out extensive study throughout the country. Therefore, the study had to be limited to some five locations of two districts of eastern Nepal on identification of and

interaction between potential soil pathogenic fungi as well as plant parasitic nematodes associated with carrot. These locations of Dhankuta and Tehrathum districts were chosen which has higher production and cultivation of carrot and vegetable crops.

CHAPTER 2

Literature review

2.1 Pathogenic soil fungi

Pythium, *Fusarium*, *Rhizoctonia* and *Sclerotinia* are the common pathogenic soil fungi associated with carrots (Domsch and Gams, 1970; Howard et al, 1978; Robertson, 1980; Mathur and Manandhar, 1993).

Pythium aphanidermatum, P. ultimum and *P. irregulare* are cosmopolitan pathogens with a wide host range (Rangaswami, 1962; Tompkins, 1975). They occur most abundantly in cultivated soils near the root region in superficial soil layers (Plaats-Niterink, 1975), less commonly in non-cultivated or acid soils where *Trichoderma* is made responsible for their absence (Barton, 1958). They are aggressive species of *Pythium*, causing damping off, root and stem rots, and blights of grasses and fruit (Lehman and Wolf, 1926). They are of economic concern on most annuals, cucurbits and grasses. *P. aphanidermatum* is considered one of the water moulds because it survives and grows best in wet soils. Warm temperatures favour the pathogen, making it an issue in most greenhouses. *P. ultimum* is one of the commonest *Pythium* species in the soil (Stanghellini and Hancock, 1970; Lumsden and Ayers, 1975; Plaats-Niterink, 1975). *P. ultimum* was also isolated from carrot by Robertson in 1980. An interesting disease of muck grown carrots, characterized by brown rot and froking, proved to be caused by *P. ultimum* (Mildenhall *et al.* 1971). *P. irregulare* and *P. sulcatum* are common soil and plant inhabiting species recorded from carrot. *P. sulcatum* was also shown to be pathogenic to carrots, causing brown discolorations, root die-back and tap-root forking (Howard and Williams, 1974; Barr and Kemp, 1976; Kalu *et al.*, 1976; Howard et al, 1978).

P. aphanidermatum has been reported during the preliminary studies of stalk rot diseases of maize in Nepal (Manandhar, 1981). Pawsey (1989) also mentioned the report of stalk rot disease in maize caused by *P. aphanidermatum*. Occurance of *Pythium* sp. has been reported in several reports (e. g. Manandhar, 1976; Pawsey, 1989); however

identification up to species level were lacking. To the extent of my knowledge there is no report of *P. ultimum, P. irregulare* and *P. sulcatum* from Nepal till date.

The distribution of *F. oxysporum* is known to be cosmopolitan from arctic tundra's (Cooke and Fournelle, 1960) to alpine pasture (Luppi-Mosca, 1960). *F. oxysporum* and its various formae speciales have been characterized as causing the various symptoms such as vascular wilt, yellows, corm rot, root rot, and damping-off. The most important of these is vascular wilt. Of the vascular wilt-causing Fusaria, *F. oxysporum* is the most important species (Agrios, 1988; Smith *et al.*, 1988). *F. solani* has a worldwide distribution especially in agricultural soil of the warmer zones and on numerous host plants (Domsch and Gums, 1970). The fungus has been recorded from rhizosphere of numerous cultivated and wild plants. They cause great reduction of root system, root rot and leaf chlorosis. *F. moniliforme* are widely distributed on numerous plants in tropical and subtropical countries and common in arable soil (Domsch and Gams, 1970; Dutta and Ghosh, 1964).

F. moniliforme has been found causing cob rot disease of maize in Nepal. This is one of the most prevalent and most destructive diseases in maize in Nepal (Manandhar, 1976; Manandhar, 1981). Pawsey (1989) also mentioned the report of *F. oxysporum, F. solani* and *F. moniliforme* associated with several crops but not with carrot. But these three fusaria were isolated from the seeds of carrot (Mathur and Manandhar, 1993). *F. moniliforme* also caused foot rot in rice (Pawsey, 1989).

Rhizoctonia solani are widely distributed and cause various types of diseases (Domsch *et al.*, 1980). They primarily attack below ground plant parts such as the seeds, hypocotyls, roots and above ground plant parts such as pods, fruits, leaves, stems. The most common disease are root rot and damping-off are world widely distributed (MacNish and Neate, 1996; Guiterrez *et al.*, 1997). The fungus occasionally infects fruit and leaf tissue located near or on the soil surface due to the mycelium and/or sclerotia. *R. solani* is a basidiomycete fungus that does not produce any asexual spores (called conidia) and only occasionally the fungus may produce sexual spores (basidiospores). These basidiospores also serve as a source for rapid and long distance dispersal of the fungus.

R. solani was found causing sheath blight in rice, banded leaf and sheath blight in maize, canker in potato, seedling disease in wheat and wire stem in cole crops in Nepal (Pawsey, 1989).

R. solani was isolated from the seeds of many crops but not from carrot (Mathur and Manandhar, 1993).

Sclerotinia sclerotiorum is a major soil borne plant pathogen. *S. sclerotiorum* infects nearly 400 plant species and causes economic damage to a wide range of crops (Abawi and Grogan, 1979; Steadman *et al.*, 1994). *S. sclerotiorum* is responsible for causing root, crown, and stem rots on various plant hosts. *S. sclerotiorum* is most prevalent in cool moist regions (Farr *et al.* 1989). The most obvious symptom is the appearance of white fluffy mycelial growth that will later produce sclerotia (Agrios, 1997). The fungus infects and produces mycelium at the base of the plant that will eventually move up the stem causing it to rot.

S. sclerotiorum was reported from stalk rot of cole crops from Nepal (Pawsey, 1989) and most prevalent in Dhankuta and Tehrathum districts (Anonymous, 2000).

2.2 Plant parasitic nematodes

Meloidogyne Heterodera, Ditylenchus, Pratylenchus, Hemicycliophor, Longidorus, Rotylenchus, Helicotylenchus are common plant parasitic nematodes associated with carrots (Santo *et al.*, 1988; Potter and Olthof, 1993; Castillo *et al.*, 1995; Siddiqi *et al.*, 1973; Hooper, 1973).

Rotylenchus uniformis is common migratory ectoparasies of roots and found associated with carrot ((Reed, 1976). They cause wilting of plant and reduction on plant yield by feeding plants' feeder fine roots and widely distributed. However, there is no report of this species in Nepal till date.

Helicotylenchus dihystera is the most widely distributed and polyphagous species in its genus. It was first described by Cobb as *Tylenchus dihystera* in 1893 from sugarcane soil, Harwood, Australia without any illustrations. Manandhar and Amatya (1987) mentioned this species from barley and wheat as reported by Mr. Sam Page from Rothamsted experimental station, U.K. in 1980.

Pratylenchus penetrans is widespread, mainly in the temperate climates (Loof, 1991) and associated with nearly 350 host crops (Mai *et al.*, 1977). They are reported from many parts of the world such as Europe, India, Japan, Philippines, Russia and USA. It is a major pest of fruit and conifer nurseries, tobacco, apple, cherry and roses (Corbett, 1973). It is associated with carrot (Kimpinski and Thompson, 1990; Potter and Olthof, 1993). *P. neglectus* is found in

temperate regions and has been reported in Europe, Canada, United States, Australia, Japan, South Africa and north-western India. They are associated with carrot (Siddiqi *et al.*, 1973). Report of these species also could not be found from Nepal.

Meloidogyne hapla has been referred to as the Northern Root-Knot Nematode because it commonly occurs in cooler environments. It is also found in the tropics and subtropics at higher elevations (Orton Williams, 1974). *M. hapla* has a wide host range with over 550 host species or varieties (Goodey *et al.*, 1965) which does not include graminaceous species. The galls induced by the nematode are usually smaller than those produced by *M. arenaria*, *M. incognita* and *M. javanica* and often have fine roots growing out of them. *M. incognita* found in the tropics and subtropics (Orton Williams, 1973) and has a wide host range with over 700 host species or varieties (Goodey *et al.*, 1965). It is never found where average monthly temperatures approaches freezing. The galls induced by the nematode are vary in size from small to large. The both species are found associated with carrot and cause more than 50% yield reduction (Wilson, 1957; Potter and Olthof, 1993). Bhardwaj (1982) and Bhardwaj and Shrestha (1983) reported *M. incognita* from Nepal.

The nematological research work in Nepal started at the end of the 1960s. During the year 1966, a heavy loss of wheat in some Terai region due to "Ear Cockle" caused by *Anguina* sp. was observed for the first time. This stimulated to carry out some research in this field. From 1967 to 1968, a preliminary survey of plant parasitic nematodes was carried out by Amatya and Shrestha for the first time in Nepal. They reported twenty-three different genera of plant parasitic nematodes including *Pratylenchus, Meloidogyne, Helicotylenchus* and *Rotylenchus* associated with many crops. In 1973, Zullini reported nematodes from high altitude in Nepal from Khumbu Himal. He described *Merlinius grandis* and *M. affinis*.

Sharma and Bhardwaj worked out the status of research on breeding crops for resistance to root-knot nematodes in 1981. They tested 43 genotypes of tomato under natural infestation and found susceptible to *Meloidogyne* spp. except Ponderosa varieties (L 1094F5-57 & 58). In 1982, Bhardwaj surveyed the root-knot nematodes of Chitwan district on 26 economic crops. Of these *Meloidogyne incognita* was the most common and predominant parasite and found in 76% of the plant specimens identified including carrot. *M. javanica* was found in 60% and *M. arenaria* in 17% of the plant samples identified. In following year, Bhardwaj and Shrestha made

more detailed survey and reported the incidence of *Meloidogyne incognita, M. javanica* and *M. arenaria* on 40 plant species in and around the Chitwan district.

In 1984, Bhardwaj and Hogger worked on root-knot nematodes and found *Meloidogyne* sp. to be important in reducing yield of many crops including carrot. He recommended the spread of improved agronomic practice and the release and multiplication of resistant varieties for nematode control.

Manandhar and Amatya (1987) enumerated 41 nematode genera and species associated with different crops in Nepal. In 1989, the same author identified 20 new host crops for *Meloidogyne incognita, M. javanica* and *M. arenaria*.

In 1989, Yadav *et al.* reported *Heliotylenchus*, *Meloidogyne* and *Pratylenchus* from the root of papaya. Rana and Ali (1992) reported the infestation due to *Meloidogyne incognita, M. arenaria* and *M. javanica* was 56%, 37%, and 23% respectively on different vegetable crops. In 1993, Pokharel reported 12 genera of nematodes with the most commonness of *Rotylenchus*, *Heliotylenchus* and *Meloidogyne*.

An overview of Nematology in Nepal was given by Manandhar and Amatya (1987) who mentioned 16 species of plant parasitic nematodes. Two more species *Hirschmanniella mucronata* and *H. oryzae* were added to this list by Pokharel (1993) (during the study of population dynamics and management of rice root and other plant parasitic nematodes from lowland rice ecosystem in Nepal). Khan *et al.* (1998) described four new species from Nepal. They were *Helicotylenchus arliani, H. alinae, Criconema nepalense* and *C. varigatum*. Later Khan and Singh (1998) added three new species; *Quininsulcium gumdariensis*, *Amphlimerlinius ekbali* and *Nagelus gerriae* from Nepal. Thus by 1998 only 25 species of plant parasitic nematodes were reported from Nepal. Pudasaini and Bert (2000) added six more nematode species *viz*: *Hirschmanniella areolata, Helicotylenchus pseudorobustus, Basiria graminophila, Tylenchorhynchus annulatus, Mesocriconema onostris* and *Hemicriconemoides strictathecatus* from Nepal.

2.3 Interaction between fungi and nematodes

Lesion nematodes, *Pratylenchus penetrans* commonly interact with other soil borne pathogens such as fungi, bacteria, and other species of nematodes. These interactions may have

no detectable effect on the host plant, the effects may be additive, or they may be synergistic (causing more damage than the combined effects of the individual pathogens).

Pratylenchus penetrans commonly interact with other soil borne pathogens such as fungi (e. g. Miller *et al.*, 1963; Oyekan and Mitchell, 1971; Jin *et al.*, 1991; LaMondia, 1999; 2003). The wounds produced by the nematode stylet during feeding provide a means of entry for a wide variety of relatively non specific soil micro-organisms, including root-infecting pathogens that may also contribute to lesion formation and may enhance the disease (Oyekan and Mitchell, 1971; Manzanilla-López *et al.*, 2004). Disease complexes due to the interaction of plant-parasitic nematodes and soil borne pathogens were reviewed but no mechanism underlying their interaction has been proven yet (Back *et al.*, 2002). *Pratylenchus penetrans* are known to interact with *Verticillium, Rhizoctonia, Helminthosporium* and *Fusarium* spp. *Pratylenchus penetrans* in combination with *Fusarium moniliformae, Rhizoctonia solani* and *Helminthosporium sativum* and *Gibberella zeae* was found in root-rot of maize in New York (Miller *et al.*, 1963). Studies have shown that *Verticillium dahliae* and *P. penetrans* can interact synergistically and cause significant yield reduction at population levels that have little or no effect when each organism is present individually (Martin *et al.,* 1982). Several studies have been carried out on a popular disease complex called 'Potato early dying syndrome' caused by the interaction of *P. penetrans* and *V. dahliae* (e. g. Rowe *et al.*, 1985; MacGuidwin and Rouse, 1990; Saeed *et al.*, 1998). The effect of *P. penetrans* on pea may be enhanced by interaction with root-infecting fungi *Aphanomyces euteiches* (Oyekan and Mitchell, 1972).

Nematodes can lower the resistance of plants to other pathogens. The resistance of peas to *Fusarium oxysporum* f. *pisi* broke down when inoculated with *P. penetrans* but not when wounded with the sterile needle; this suggests that the nematode does more than just wounding the host and letting the fungus in (Oyekan and Mitchell, 1971). Wilt incidence increased in tomato when *Verticillium albo-atrum* occurred with *P. penetrans* (Conroy *et al.*, 1972). In an experiment, *P. penetrans* alone or in combination with the black root rot pathogen, *Rhizoctonia fragariae,* reduced strawberry yield; the interaction of the two pathogens was additive rather than synergistic (LaMondia, 1999). Weakened or dying cells in strawberry roots caused directly or indirectly by *P. penetrans*, might be more susceptible to *R. fragariae*, leading to increased disease (LaMondia, 2003). In a further study, *P. penetrans* increased *R. fragariae* infection and

the severity of black root rot by 50% (LaMondia and Cowles, 2005). There was an additive effect on growth reduction of red clover caused by *P. penetrans* and *Fusarium avenacearum* and an enhanced recovery of *P. penetrans* from soil and roots of *Fusarium*-infected plants (Jin *et al.*, 1991).

Lesion nematodes are known to interact with *Verticillium* and *Fusarium* fungi, which cause wilt diseases on pepper, eggplant, tomato, potato and numerous other plants. Lesion nematodes also interact with *Trichoderma viride* on alfalfa and celery. Such disease complexes may kill plants, whereas nematodes alone seldom cause such a severe reaction.

There is strong interaction between *Meloidogyne* and fungi. When bean is infected with *Uromyces phaseoli* and *M. incognita*, the reproduction of both parasites will reduce (Bookbinder and Bloom, 1980). The *Meloidogyne-Fusarium* interaction has been extensively studied because of its significance on major crops such as cotton (Sasser, 1972) and tobacco (Milne, 1972). The presence of *Meloidogyne* cause to produce some factors which break the resistance of host against *Fusarium*.

Combined infections of host plants with nematodes (*Belonolaimus, Meloidogyne, Pratylenchus* and *Heterodera* spp.) and species of *Pythium* often had greater effects than either of these organisms alone (Littrell and Johnson, 1969; Nichols *et al.*, 1964; Roman and Koike, 1970; Santo and Holtzmaww, 1970; Starr and Aist, 1977; Whitney, 1974). *Pythium irregulare* and *P. ultimum* were reported to cause root dieback diseases and thereby forking in carrot (Liddell *et al.*, 1989; Davis and Nunez, 1999, Mildenhall *et al.*, 1971). *P. penetrans* are also associated with carrot where these fungi are present. Root infection by *P. penetrans* reduced carrot growth, but soil inoculation with *Glomus sp*. spores compensated for the damage caused by *P. penetrans and* reduced *P. penetrans* soil densities by 49% (Talavera *et al.*, 2001).

However to the extent of my knowledge there is no study on the interaction between *Pratylenchus penetrans* and *Pythium* spp. The study on the interaction between nematodes and fungi is the first of its kind in Nepal.

2.4 Overview on *Pythium* spp.

The mycelium of *Pythium* species is colourless, sometimes lustrous, occasionally slightly yellowish or greyish lilac. The hyphae are hyaline, cross septa are lacking except in old, mostly empty hyphae or where they delimit reproductive organs. Protoplasmic streaming is often clearly

visible in young hyphae. The production of aerial mycelium is more or less dependent on the medium used. On cornmeal and potato-carrot agars most species do not produce aerial mycelium, but on oatmeal agar several species develop profuse aerial mycelium. For the evaluation of the mycelial development it is necessary to know the medium on which the fungus has been cultivated.

The asexual reproduction takes place by means of zoosporangia and zoospores. In *Pythium* the zoospores are not formed in the sporangium itself but in a vesicle outside it. The sporangium is separated from the rest of the mycelium by a cross wall. The sporangia can be filamentous or more or less spherical. The sexual reproduction takes place by means of oogonia and antheridia (see Fig. 2.1). The female organs, the oogonia, are spherical to limoniform and are intercalary or terminal. The oogonial wall can be smooth or ornamented with projections. The antheridia, the male organs, consist of an antheridial cell which can be sessile on a hypha, intercalary or formed terminally on an antheridial stalk. The antheridial cell touches the oogonium and forms a fertilization tube which penetrates the oogonium. Only in rare cases more than one oospore is produced inside an oogonium. The thickness of the oospore wall is characteristic for the species.

After maturation of the oospore, a dormant phase is usually necessary, before germination can take place. At germination, the oospore is converted into a thin-walled structure, which produces a germ tube or acts as sporangium and forms zoospores (De Bary, 1881; Ayers and Lumsden, 1975). The first stage in the germination of oospores is the absorption of the endospore, which is dependent on an exogenous calcium supply. In many *Pythium* species undifferentiated hyphal swellings are formed which can also germinate and form a new thallus. In most cases they cannot be distinguished from young spherical sporangia or oogonia. Hyphal swellings may be intercalary or terminal.

In many species of *Pythium* sickle-, club- or sausage-shaped or subglobose structures of various sizes are formed which are called appressoria (Agnihotri 1969). These appressoria may be single or connected to each other in chains or clusters. On a host plant they can form distal or lateral infection pegs. In cultures the appressoria are attached to the walls of the Petri dishes.

Species of *Pythium* can live saprophytically or parasitically. Their parasitic role often depends on external factors. When conditions are favourable for the fungus but less for the host, *Pythium* species can become very pathogenic and cause rot of fruit, roots or stems, pre- or post-

emergence damping-off of seeds and seedlings. Young or watery tissue is preferentially attacked. Infection takes place when zoospores produce germ tubes (Spencer and Cooper, 1967) or hyphal elements form appressoria and then penetrate the plant by means of infection pegs (Miller *et al.*, 1966). Sufficient amounts or excess of soil water often favour infection and the severity of

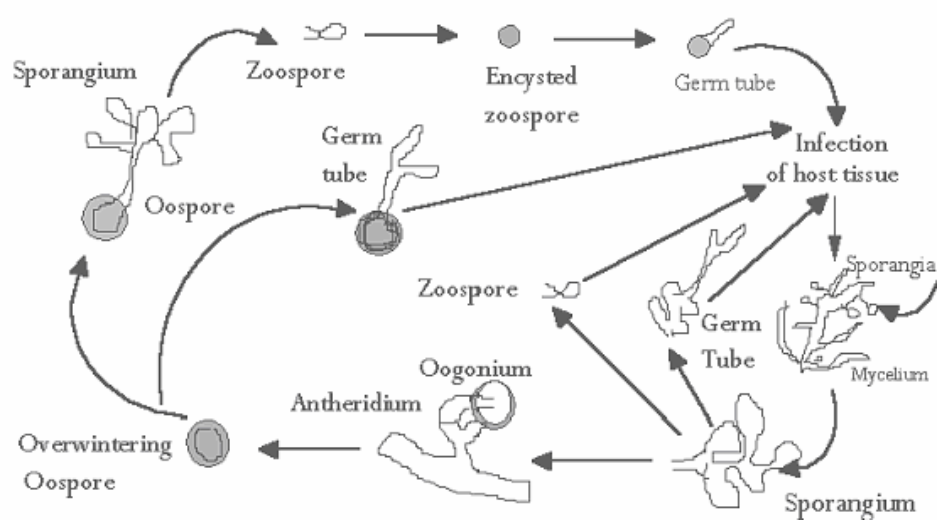

Fig. 2.1 Life cycle of *Pythium* spp. (e.g. *P. aphanidermatum*) (from Plaats-Niterink, 1981).

attack. Infection mostly takes place on the young roots of phanerogams, but leaves can also be affected. In susceptible plants, root exudates can cause an accumulation of zoospores and accelerate their encystment and germination (Tripathi and Grover, 1978), especially in differentiating or injured roots (Kraft *et al.*, 1967). Resistance of plants to *Pythium* species may be related to the presence of inhibitory phenolic compounds (Chakravarty and Srivastava, 1967), as well as to other substances (Kraft, 1974).

2.5 Overview on *Pratylenchus* spp.

Small nematodes measuring less than 1 mm with prodelphic sexual system of female. *Pratylenchus* spp. has a simple life cycle (Fig. 2.2). It reproduces sexually (Hung and Jenkins, 1969; Thistlethwayte, 1970) and after fertilization, females lay eggs singly inside the host root or in soil. The first moult occurs in the egg; second-stage juveniles hatch from the egg and moult three times more, between feeding intervals, to become adult. The life cycle may take 30 to 86

days depending upon species and temperature. Generally, fourth stage juveniles, adults (Miller, 1968; Kable and Mai, 1968) and eggs are the main over-wintering stage (Dunn, 1972). All mobile stages of both sexes invade roots but most is done by 4th stage juveniles and adults (Sonitirat and Chapman, 1970).

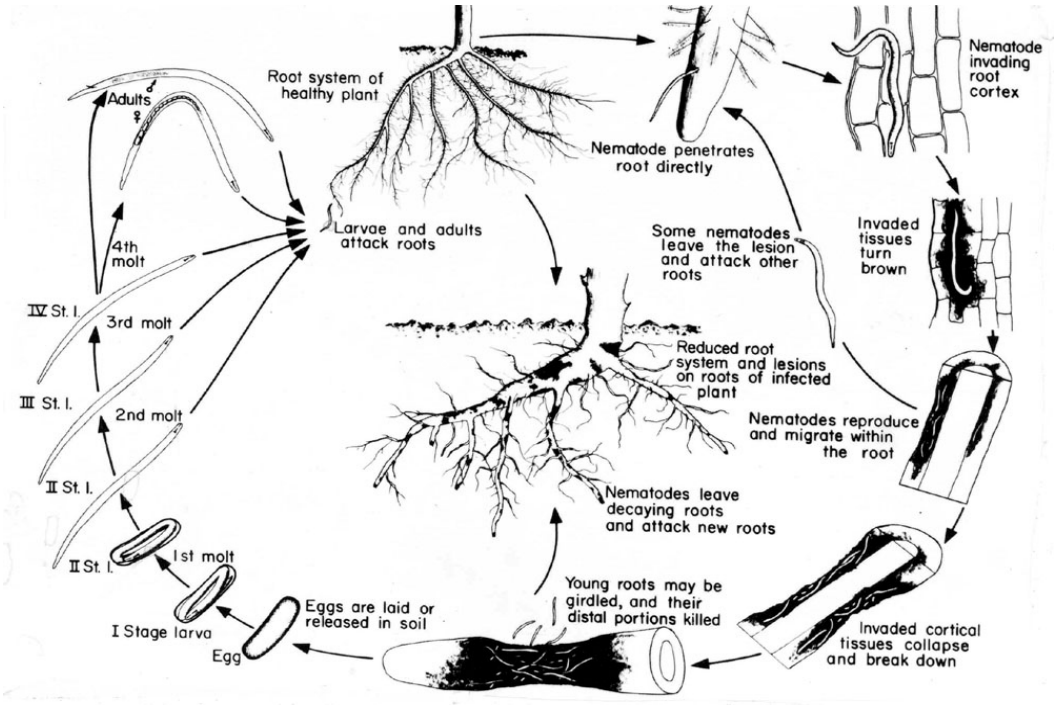

Fig. 2.2 Life cycle of root lesion nematode (*Pratylenchus* sp.) in the root tissue (http://ucdnema.ucdavis.edu/imagemap/nemmap/ent156html/nemas/pratype)

Pratylenchus spp. enters the roots, feeds on cortical cells periodically and migrates through the dead cell. Later, cells in the cortex break down and cavities are formed; affected roots may show lesions (e.g. Oyekan *et al.*, 1972; Townshend and Stobbs, 1981; LaMondia, 2003). In later stages of attack, it may penetrate and damage the vascular tissues (Mamiya, 1970; Acedo and Rohde, 1971). The above ground part of affected plants are usually stunted and chlorotic, with early death of old leaves or die-back of twigs in woody plants (e.g. Hoestra and Oostenbrink, 1962).

CHAPTER 3

Methods and Materials

3.1 Description of the study area

Nepal is situated at 80^{o} 30' E to 88^{o} 10'E longitude and 26^{o} 20'N to 30^{o} 26'N latitude. The study was carried out in two eastern districts of Koshi zone: Dhankuta and Tehrathum. Dhankuta measures about 891 sq. km in area with 609 to 2438m elevation above sea level and inhabited by 166479 people (2001). Tehrathum measures about 679 sq. km in area with 345 to 2962m elevation above sea level and inhabited by 113111 people (2001). Tehrathum is adjacent Dhankuta in northern east side. Both districts are then neighbouring with Panchthar district in the east, Taplejung and Sankhuwasabha districts in north, Bhojpur and Udayapur districts in west and Sunsari and Morang districts in south (Fig. 3.1).

Samples were taken from three spots from Dhankuta district: Sindhuwa, Belhara and Jeetpur with elavation of 2250m, 1600m and 1650m above sea level respectively and located around 26^{o} 55' N longitude and 87^{o} 40' E latitude. While samples in Tehrathum were collected from two spots: Basantapure and Sukkrabare with elavation of 2400m and 1900m above sea level respectively and located around 26^{o} 58' N longitude and 87^{o} 44' E latitude.

Agriculture is the only major source of economy for these people. These five areas were chosen because of higher prevalence of diseases which is limiting the production of carrot. Besides carrot, cole crops, maize, potato, radish are other crops grown in these areas. These areas are highly potential area for the production of carrot and cole crops which are exported to India (Anonymous, 2000).

3.2 Sampling

Both root and soil samples had collected from different carrot growing areas from Dhankuta and Tehrathum district of eastern Nepal as shown in Fig. 3.1 and Table 3.1. From each location, 10 sub-samples up to 30 cm deep around the carrot rhizospheres were taken randomly

and mixed together to form one composite sample. Each composite sample (≈1 kg) was air tightly packed in a plastic bag. The bags were labelled with details of location, soil type and date of sampling. All the samples were sent to laboratory and were kept in the refrigerator until it was extracted. The characteristics of field are presented in Table 3.1.

TABLE 3.1 Characteristics of field where samples were taken.

District	Location	Soil type*	Soil pH	Altitude
Tehrathum	Basantapure	Loamy sand	5.5	2400 masl**
	Sukkrabare	Loamy sand	5.2	1900 masl
Dhankuta	Sindhuwa	Loamy sand	5.2	2250 masl
	Belhara	Loamy sand	5.6	1600 masl
	Jeetpure	Loamy sand	5.5	1650 masl

* Soil type according to soil triangle of soil classification system of USDA.

* *meters above see level.

3.2.1 Extraction

The optimal procedures which are required for the extraction of candidate genera of fungi and nematodes from root and soil were employed. These fungi genera can have interaction with plant parasitic nematodes in the disease severity of carrot.

3.2.2 Extraction of fungus from root samples

3.2.2.1 Isolation of *Pythium* spp.

The roots of carrot from every sample was first rinsed in tap water, then rinsed in sterile water and finally blotted dry on sterile paper towels. The root pieces were transferred onto the selective medium for *Pythium* spp. in Petri plates in PVPP agar containing pimaricin, vancomycin, penicillin and pentachloronitrobenzene as described by Liddell *et al.* (1989). The antibiotics in these media limit bacterial contamination and pimaricin prevented the growth of *Phytophthora* species. The recipes of all media used in the study are given in appendix.

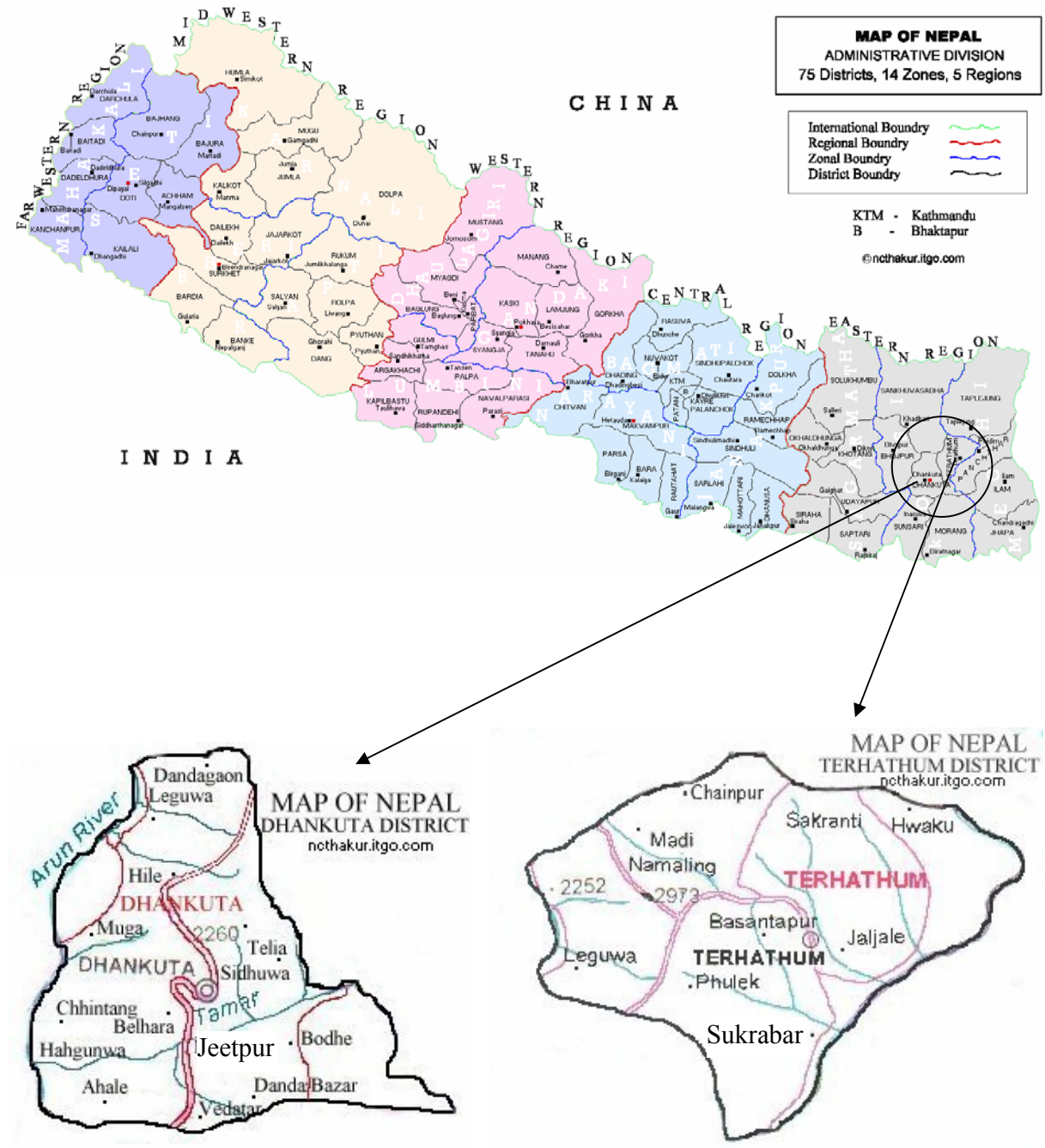

Fig. 3.1 The location where sample was taken: Sindhuwa, Belhara and Jeetpur in Dhankuta district and Basantapur and Sukrabare in Terhathum district are shown in the maps.

3.2.2.2 Isolation of *Fusarium* spp.

The roots of carrot from every sample was first rinsed in tap water, then rinsed in sterile water and finally blotted dry on sterile paper towels. The root pieces were transferred onto the selective medium for *Fusarium* spp., the dichloran chloramphenical peptone agar (DCPA) as described by Burgess *et al.* (1988) in Petri plates. The plates were kept in diffuse light for incubation at 25°C as light kills *Fusarium*.

3.2.2.3 Isolation of *Rhizoctonia* spp.

Small samples of carrot roots (0.5 cm of length) were cut, rinsed in tap water, rinsed in sterile water and finally blotted dry on sterile paper towels. Roots were transferred to a general alkaline water agar medium. In order to isolate the fungus from the host, sclerotia were collected from infected plant parts and air dried. Surface sterilised the sclerotia by submerging each peace of sclerotia in a Petri dish containing 1% Sodium hypochlorite for ten minutes. Following this, the sclerotia were momentarily dry on tissue paper before transfer to the centre of a Petri dish containing 1% water agar. After transferring the sclerotia to the centre of Petri dish, Petri dish were sealed with parafilm, inverted and placed in an incubator at 15 ± 2°C for 7 days (Sneh *et al.*, 1996).

3.2.2.4 Isolation of *Sclerotinia* spp.

Sclerotinia spp. was isolated from host tissue. In order to isolate the fungus from the host, sclerotia were collected from infected carrot parts and air dried. Sclerotia were allowed to germinate by placing on moist filter paper, surface-sterilized with NaOCl or CaOCl, sectioned aseptically, and plated on PDA. To isolate *Sclerotinia* spp. from diseased tissue, sections were incubated in a black plastic bag with moist filter paper at 20°C (Pratt, 1992). After several days hyphae were then transferred to PDA and incubated at room temperature.

3.2.3 Extraction of fungus from soil samples

3.2.3.1 Isolation of *Pythium* spp.

Air-dried 20 mg soil from every sample was spread over the same selective medium as mentioned above (3.2.1.1) in Petri plates. A sterile, bent glass rod was used to disperse the sample over the entire plate. The plates were kept upside down and read in 24 hours.

3.2.3.2 Isolation of *Fusarium* spp.

Isolation of *Fusarium* spp. from soil samples were carried out in the same media described for the isolation of *Fusarium* spp. from root samples in 3.2.1.2.

3.2.3.3 Isolation of *Rhizoctonia* spp.

Isolation from soils was done by soil sieving to collect sclerotia. Sieves with openings of 0.43-2.00 mm were used to collect sclerotia (Sneh *et al.*, 1996). Sclerotia were allowed to germinate by placing on moist filter paper as described in 3.2.1.3.

3.2.3.4 Isolation of *Sclerotinia* spp.

Isolation from soils was done by soil sieving to collect sclerotia. Sieves with openings of 0.43-2.00 mm were used to collect sclerotia (Pratt, 1992). Sclerotia were allowed to germinate by placing on moist filter paper as described in 3.2.1.4.

3.2.4 Extraction of nematodes from root samples

3.2.4.1 Extraction of migratory endoparasitic nematode spp.

Roots were separated from soil by hand picking and sieving them through 1mm sieve. All the roots were collected from each sample separately. Roots were washed in tap water and were chopped into 0.5-1 cm pieces and macerated in high speed warring blender for 1 min to allow the release of nematodes into water. After that nematodes were extracted from the macerated root suspension by using zonal centrifugal flotation methods (Pudasaini *et al.*, 2006). Nematode suspension obtained from this method contains $MgSO_4$ solution. To remove excessive $MgSO_4$ solution, nematodes that were collected in 150 ml glass beaker were allowed to settle down. After that the water was pipette out. Then distilled water was added and kept in refrigerator at 4°C.

3.2.4.2 Extraction of sedentary endoparasitic nematode spp.

For sedentary nematodes root galls were examined and visible root galls, if present, were picked up with the help of forceps and collected in small plastic Petri-disc in few drops of water. If females were present, they were collected with the help of forceps and kept in embryo-disc. If egg masses were present, they were dissolved in sodium hypochlorite solution (NaOHCl) (5.2

%) for 4 minutes with occasional agitation to free the eggs which were later incubated to allow hatching.

3.2.5 Extraction of nematode from soil samples

Nematodes present in the mineral fraction of soil sample from each locality were extracted by using zonal centrifugal flotation methods (Pudasaini *et al.*, 2006) as state in 3. 2. 3. 1 and also store in the same way.

3.3 Processing and mounting

3.3.1 Processing and mounting of fungi

Stain solutions were used for the observation of morphological characteristics of micro-organism. Various dyes can be used to stain micro-organism to make them clearly visible. Mostly lactophenol and cotton blue were used for the staining of the fungi. To preserve the slide the coverslip was sealed with nail polish (for detail see Appendix).

3.3.2 Processing and mounting of nematodes

Killing, fixing and processing of nematodes was done by Glycerol-ethanol solution as described by Seinhorst (1959) and modified by De Grisse (1969). Nematodes were mounted in anhydrous glycerine on aluminium slides with double cover slips (Cobb, 1917) as described by De Grisse (1969). A copper tube (1.5 cm diameter) was heated, dipped in paraffin was and placed in the middle of a rectangular cover slip forming a paraffin ring. Then 5-10 nematodes were transferred in a small drop of anhydrous glycerine in the centre of paraffin ring. The round cover slip was put over the ring and heated on a horizontal plate to melt the paraffin wax (for detail see Appendix).

3.4 Identification

3.4.1 Identification of fungi

The fungi were identified based on morphology and morphometrics. Drawings were made by using light microscope (Wild Heerbrugg, M12, Swiss made). All drawings were made in 1000x magnification to get better view. Morphometrics were taken using an Olympus BX50

compound microscope equipped with Leica image-capture IM500 system and software. The following abrreviations were used in the text for morphometrical data. All morphometrical data were presented in mean with standard deviation.

HyW	Width of hyphae
HySW	Width of hyphal swelling
SpoW	Width of sporangia
SpoL	Length of sporangia
ZosD	Diameter of zoospore
ZosL	Length of zoospore
OogW	Width of oogonium
AntW	Width of antheridium
AntL	Length of antheridium
OosD	Diameter of oospore
OosWTh	Thickness of oospore wall
MicD	Diameter of microconidium
MicL	Length of microconidium
MacD	Diameter of macroconidium
MacL	Length of macroconidium
ChlD	Diameter of chlamydospore

The detail morphological as well as morphometrical characters can be achieved only by culturing fungi in the selective media required for the identification of particular genus or species which is described below (for detail method, see in Appendix).

3.4.1.1 Identification of *Pythium* spp.

Identification of *Pythium* spp were done on the basis of production of sporangia and discharge of zoospores and the morphology and morphometrics of oogonia, antheridia, sporangia and the colony growth on potato-carrot agar (PCA) by using the key of Plaats-Niterink (1981).

3.4.1.2 Identification of *Fusarium* spp.

Identification of *Fusaria* spp were done on the basis of morphology and morphometrics of microscopical characters such as microconidia, macroconidia and chlamydospores and colony morphology using the Fuskey (Seifert, 1996).

3.4.1.3 Identification of *Rhizoctonia* spp.

The anatomy of the septal pore and the cellular nuclear number (CNN) were used to differentiate the species of *Rhizoctonia* fungi.

3.4.1.4 Identification of *Sclerotinia* spp.

Sclerotinia spp. is best identified by the observation of the sclerotia, ascoma, colony morphology and finally by using key (Hanlin, 1998).

3.4.2 Identification of nematodes

The nematodes were identified based on morphology and morphometrics. Drawings were made by using light microscope as described in 3.4.1. The following symbols were used in the text for morphometrical data according to Geraert (1968) and Geraert and Raski (1987). All morphometrical data were presented in mean with standard deviation.

N	Number of specimens
L	Total body length
a	Total body length/maximum body length
b	Total body length/distance from anterior end to junction of pharynx and intestine
b’	Total body length/distance from anterior end to posterior end of pharyngeal glands
c	Total body length/tail length
c’	Total tail length/anal body width
b”	Total body length/distance from anterior end to middle of median bulb
V	Distance from anterior end to vulva × 100/ total stylet length
V’	Distance from anterior end to vulva × 100/ distance from anterior end to anus
M	Length of anterior conical part of stylet × 100/total stylet length
O	Distance from stylet base to dorsal gland outlet × 100/total stylet length
MB	Distance of median bulb from anterior end × 100/total pharyngeal length
VL/VB	Distance between vulva and posterior end of body/vulval body width

Tail	Tail length
Abw	Anal body with
Stylet	Length of stylet
SbW	Width of stylet base
DGO	Distance between stylet base and dorsal gland orifice
mb	Length from anterior end to middle of median bulb
Nr	Length from anterior end to nerve ring
E. pore	Length from anterior end to excretory pore
R Ph	Number of annuli from anus level to phasmid level
DOG	Dorsal pharyngeal gland
PVUS	Post vulval uterine sac
Bb L	Length of basal bulb
Bb W	Width of basal bulb
Gub.	Gubernaculum length
W	Width of body (for root-knot- and cyst forming nematodes)
h	Length of hyaline part of tail
MbL	Length of median bulb
MbW	Width of median bulb
MbVL	Length of median bulb valve
MbVW	Width of median bulb valve

Identification of root-knot nematodes were done by studying perennial patterns. Preparation of perennial patterns is given in detail in Appendix.

3.5 Estimation of population density of fungi and nematodes

First of all the composite samples were well mixed and 100 gm soil sample for the extraction of fungi and another 100 gm soil sample for the extraction of nematodes were taken from each sample. Roots were separated from soil by hand picking and sieving them through 1mm sieve as described above. The roots were ringed afterward. The roots obtained from the sample for the extraction of fungi were divided into several small equal portions. Finally each small portion of root sample and one gram soil sample (after removing the roots) were subjected

to the serial dilution (10^{-5} to 10^{-7}) and plated on the selective media (described above) for the determination of population densities. In case of nematodes, rinsed root obtain from 100 g soil were macerated and nematodes were extracted by using zonal centrifuge machine from both macerated root and soil (after the removal of root) separately, as described above. Finally, population densities present in each 100 gm soil sample were expressed as colony forming unit (CFU) for fungi and individual number for nematodes.

3.6 Interaction of fungi and nematodes

3.6.1 Inoculum preparation

For the interaction study of fungus and nematode, only one combination was chosen. *Pythium ultimum* as candidate fungal species and *Pratylenchus penetrans* as a candidate nematode species were chosen. Both pathogens were cultured to raise the inoculum to carry out growth chamber study.

3.6.1.1 Inoculum preparation of fungi

The pure culture of *Pythium ultimum* maintained on potato-carrot agar (PCA) was used to raise inoculum. *P. ultimum* isolated from carrot roots collected from Sinduwa (Dhankuta district) were cultured on maize meal-sand medium (98% washed autoclaved quartz sand, 2% maize meal by weight with 20% water by volume) for 2 week at 25°C in the dark. The colonized medium was then mixed with washed autoclaved sand (1:1, w/w), incubated for 2 days at room temperature and diluted with 0.2% water agar at 1:50 and 1:200 (w/v) onto PVPP agar to estimate the number of colony forming unit (CFU) per gram of sand. The inoculum-sand mixture was then further diluted with washed autoclaved sand to obtain desirable cfu/g sand. Thus obtained population density was used in all subsequent experiments.

3.6.1.2 Inoculum preparation of nematodes

The pure culture of *Pratylenchus penetrans* collected from Sinduwa (Dhankuta district) were was maintained on carrot root disc *in vitro* for three months to raise starting density to rare in huge number. *Pratylenchus penetrans* was reared on maize (*Zea mays* L., cv. Husar) grown in the glasshouse at 22 ± 5°C in a sandy loam soil (sand 87.4%; loam 8.6%; clay 4.0%) with pH 6.2 and 2.5% organic matter. Soil was sterilized by heat (100°C) during 12 hours, after which the

soil was spread out for 1.5 months to eliminate toxins and to allow re-colonization of micro-organisms from the air. Three maize plants were grown per 4-liter polyethylene pot (20 cm diameter). Every week, soil moisture content in the pot was adjusted to 15% by weight (oven dry weight). A pure culture of *P. penetrans* obtained from carrot root discs was inoculated on 15-day old maize plants. Maize was fertilized with commercial fertilizer at a rate of 580 kg/ha (N:P:K ratio=12:7:15) once just before sowing and after one month. After three and half months, the roots were harvested by sieving the soil, chopped into 0.5 to 1-cm parts and kept in a mist chamber (Seinhorst, 1950) for two weeks. Every four days nematodes were collected from the mist chamber and stored at 4°C. Later on nematodes were transferred onto a cotton-wool filter (Oostenbrink, 1960) for 24 hours to remove remaining root debris. Finally, pure clean nematodes were surface sterilized by keeping them in streptomycin sulphate solution (2000 ppm) for 24 hours and rinsed three times with deionised water. The density of *P. penetrans* per ml water was calculated in stock nematode suspension.

3.6.2 Growth chamber condition

The experiments about the interaction of *Pythium ultimum* and *Pratylenchus penetrans* on disease development on carrot were established in the growth chamber. The temperature and relative humidity were adjusted at 20°C and 70% respectively for both day and night. The intensity of light was $320\mu Em^{-2}s^{-1}$. The photoperiod of 14 hours day and 10 hours night was fixed.

3.6.3 Experimental design

The experiment design or layout was as follow: (i) nematode only (N); (ii) first nematode inoculum and 2 weeks later fungus inoculum (NF), (iii) first fungus inoculum and 2 weeks later nematode inoculum (FN), (iv) fungus inoculum only (F) and (v) control without any pathogen (C). Three nematodes and 200 cfu of fungus per gram of soil were used as inoculum densities which were previously reported to cause disease.

3.6.4 Recipient selection

In order to give full volume of space and substrate a pot size already determined to mimic the field condition for carrot (Pudasaini *et al.*, unpubl.) were used. A polyvinylchloride (PVC) pipes of volume 621 cm^3 (22 cm height and 6 cm diameter) were used. PVC pipes were sealed

with polythene bag on the bottom and filled with 830 gram autoclaved quartz (particle size 150-212 µm) sand. In case of treatment F, NF and FN some sand along the pipe length were removed with the help of aluminium tube (diam. 1 cm) at three spots and immediately wooden rods of similar diameter were inserted into these channel left by aluminium tube in order to keep space to inoculate fungus.

3.6.5 Plant preparation and inoculum

Seeds of carrot were surface disinfested for 1 min in 1.0% sodium hypochlorite in a 10% aqueous ethanol solution. Seeds were sown in 1-2 cm deep in PVC pipes (Fig. 3.2). Five days after germination, plants were thinned to single plant per pot. Plants were allowed to grow in the pots for the 2 weeks. For the treatment N and NF nematodes were inoculated into the pots at the rate of 3 nematodes per gram soil. Nematode inoculums were done by injecting nematode suspension with the help of veterinarian glass syringe. For the treatment C, only water was injected into the pots. For the treatment F and FN, maize meal-sand medium with fungus at the rate of 200 cfu/ g of soil were inoculated in the space left after the removal of wooden rod. Two weeks after the first inoculum, the second inoculum to the treatment NF were done by

Fig. 3.2 Carrot grown in polyvinylechloride (PVC) pipes

inoculating fungus with the same rate and procedures as described above. In case of treatment FN, the second inoculums with nematodes were done with similar rate and procedures. Every treatment was replicated 10 times in all experiments and randomised in block design in growth chamber (Fig. 3.2). The experiments were left for 80 days after first inoculum in order to complete double generation of the nematode after inoculation.

3.6.6 Irrigation and fertilization

Watering was done every alternate day. The moisture level kept around 15 %. Liquid fertilizer 'Substrail' (4:5:6 % N:P:K with other trace elements) was prepared at 7 ml/litre of water and given at the rate of 2.5 ml/50 gram of sand twice a week for carrot.

3.6.7 Estimation of damage

After 80 days, plants were harvested and fresh as well as dry weights were determined. Qualitative data were also recorded. Lesions on the carrot tap roots were also recorded. Dry weights were determined after drying whole plant in the hot air oven at 70°C for 72 hours.

3.7 Statistical analysis

The dry weights of whole plants in gram were subjected to the analysis of variance (ANOVA) by using SPSS statistical package. Means were separated by Duncan's multiple range tests ($p \leq 0.05$). Means were also compared with independent sample t-test ($p \leq 0.05$).

CHAPTER 4

Results

4.1 Species composition and distribution

Species composition of both pathogenic soil fungi and plant parasitic nematodes in the samples collected from different localities were present in the Table 4.1. Four genera and nine species of fungi and four genera and six species of nematodes were identified. Among fungi, four species of *Pythium*, viz. *Pythium aphanidermatum, P. ultimum, P. irregulare, P. salcatum* three species of *Fusarium,* viz. *Fusarium oxysporum, F. solani, F. moniliforme* one species of *Rhizoctonia solani* and *Sclerotinia sclerotiorum* were identified (Table 4.1).

TABLE 4.1 Species composition of pathogenic soil fungi and plant parasitic nematodes present in the samples collected from different location of Dhankuta and Tehrathum districts.

		District/Location									
		Tehrathum				Dhankuta					
		Basantapure		Sukkrabare		Sindhuwa		Belhara		Jeetpure	
	Pathogens	Root	soil	root	soil	root	soil	root	soil	root	soil
Fungi	*Pythium aphanidermatum*	-	-	-	-	-	-	-	+	+	+
	P. ultimum	+	+	+	+	+	+	+	+	+	+
	P. irregulare	+	+	+	+	+	+	+	+	+	+
	P. salcatum	+	+	+	+	+	+	-	+	-	-
	Fusarium oxysporum	+	+	+	+	+	+	+	+	+	+
	F. solani	-	-	+	+	+	+	+	+	+	+
	F. moniliforme	+	+	+	+	+	+	+	+	+	+
	Rhizoctonia solani	+	+	+	+	+	+	+	+	+	+
	Sclerotinia sclerotiorum	+	+	+	+	+	+	+	+	+	+
Plant-parasitic nematodes	*Pratylenchus penetrans*	+	+	+	+	+	+	+	+	-	-
	P. neglectus	+	+	-	-	+	+	+	+	-	-
	Meloidogyne hapla	+	+	+	+	+	+	+	+	+	+
	M. incognita	-	-	+	+	-	-	+	+	+	+
	Helicotylenchus dihystera	-	+	-	+	-	+	-	+	-	+
	Rotylenchus uniformis	-	-	-	+	-	+	-	+	-	+

Among nematodes, two species of *Pratylenchus,* viz. *Pratylenchus penetrans, P. neglectus*, two species of *Meloidogyne* viz. *Meloidogyne hapla, M. incognita* one species of *Helicotylenchus dihystera* and *Rotylenchus uniformis* were identified (Table 4.1).

Population structure, distribution and densities of both pathogenic soil fungi and plant parasitic nematodes in the samples collected from different locations were visualised in graphical diagrammes.

4.1.1 Distribution of fungal species

The colony forming unit (CFU) of different fungal species per gram dry soil sample were determined and presented in percentage for different species in each location (Fig. 4.1). *Fusarium* species were the highest among all species studied in all locations. In colder climate *Furarium oxysporium* was the highest i.e. 74% in Basantapur, 56% in Sinduwa while in warmer climate *Furarium solani* was the highest i.e. 44% in Jeetpur, 37% in Belhara. *Furarium moniliforme* occured at maximum 38% in Jeetpue and minimum 14% in Basantapur (Fig. 4.1). In all samples, *Fusarium* species were most abundant except the absence of *Furarium solani* in Banastapur.

Among the *Pythium* species, *Pythium irrugularis* and *P. ultimum* were present in all samples, with the highest *P. ultimum* in Basantapur with 5% and lowest in Jeetpur with 1%. *P. aphanidermatum* was recovered only from Belhara and Jeetpur which was less than 1% (Fig. 4.1). Occurrence of *Rhizoctonia solani* and *Sclerotinia sclerotiorum* were less than 1%.

The presence of CFU/g dry soil sample of all fungi in all samples was also presented in number in bar diagramme (Fig. 4.2). The numerical value is converted in log number in order to obtain best visualization of species that occurred in lower number.

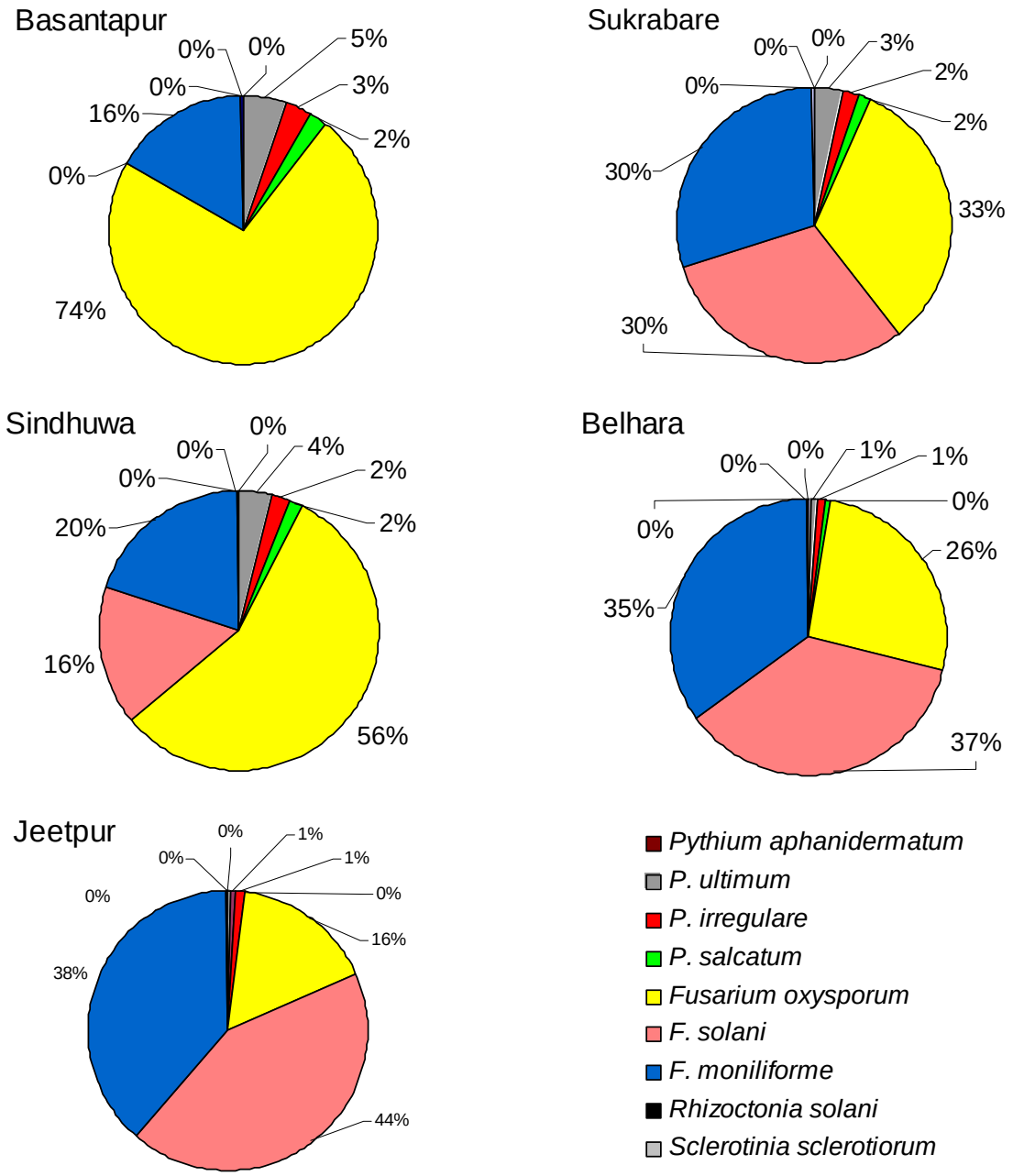

Fig. 4.1 Colony forming unit (CFU)/ g dry soil sample of different fungal species occurs in different location of Tehrathum and Dhankuta district. Data are present in percentage.

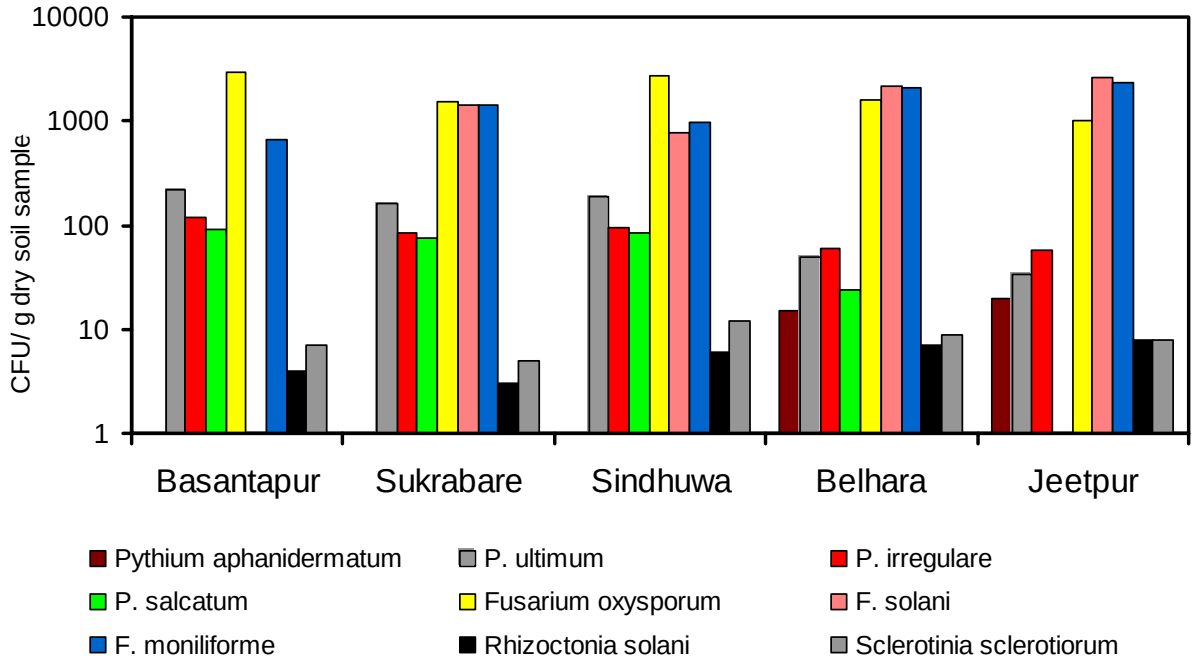

Fig. 4.2 Colony forming unit (CFU)/ g dry soil sample of different fungal species occurs in different location of Tehrathum and Dhankuta district. Data are present in log numbers.

4.1.2 Distribution of nematode species

The number of different nematode species per 100 gram dry soil sample were determined and presented in percentage for different species in each location (Fig. 4.3). *Pratylenchus* species were the highest among all species studied. *Pratylenchus penetrans* were highest in Sindhuwa with 62%, but absent from Jeetpur. *Pratylenchus neglectus* was highest in Belhara with 27% but absent from Sukrabare and Jeetpur (Fig. 4.3).

Meloidogyne hapla was present in all samples with the highest percentage in Sukrabare with 17% and lowest in Belhara with 10%. But when it is compared between locations, it was 4.3 nematode/ gm dry soil sample in Sindhuwa and 1.1 nematode/ gm dry soil sample in Jeetpur. *Meloidogyne incognita* was highest in Jeetpur with 36% and lowest in Sukrabare with 11%. But absent from Basantapur and Sinduwa (Fig. 4.3).

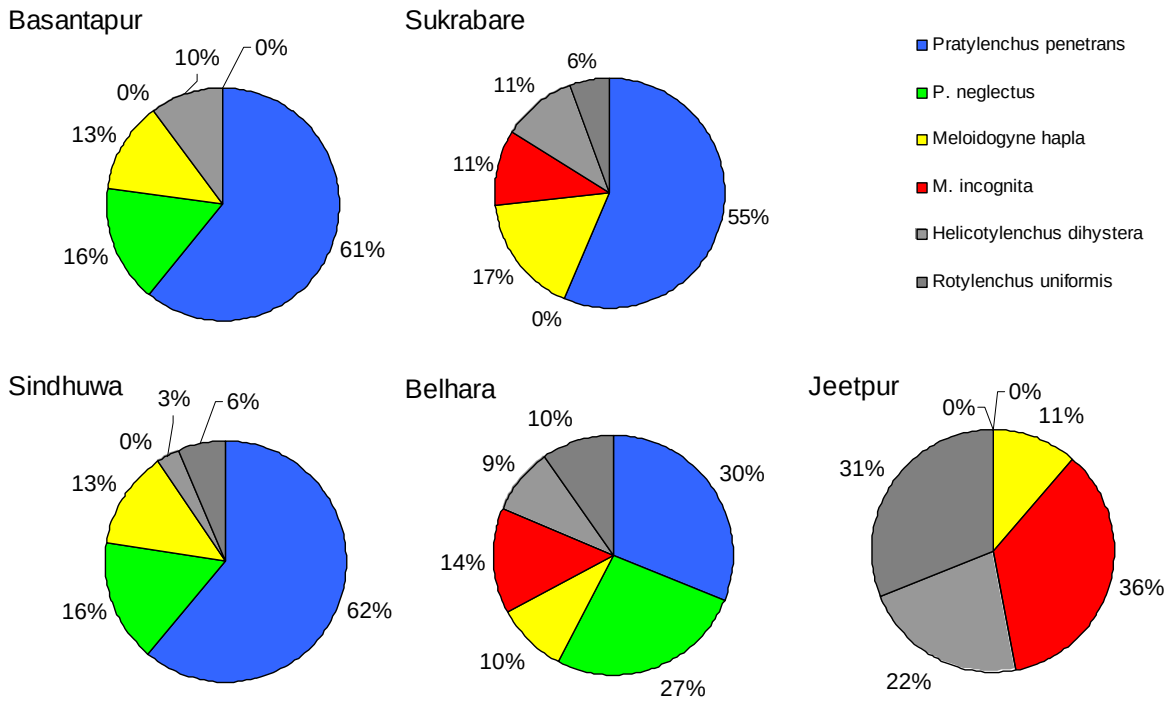

Fig. 4.3 Nematodes/gm dry soil sample in different location of Tehrathum and Dhankuta district. Data are present in percentage.

Helicotylenchus dihystera was present in all samples with highest in Jeetpur (22%) and lowest in Sinduwa (3%). *Rotylenchus uniformis* was highest in Jeetpur (31%) and lowest in Sukrabare (5.5%) (Fig. 4.3).

The nematode number/gram dry soil samples were also presented in number in bar diagramme (Fig. 4.4). When density of species was compared between locations, Sindhuwa showed the highest density of *Pratylenchus penetrans*, *P. neglectus* and *Meloidogyne incognita.*

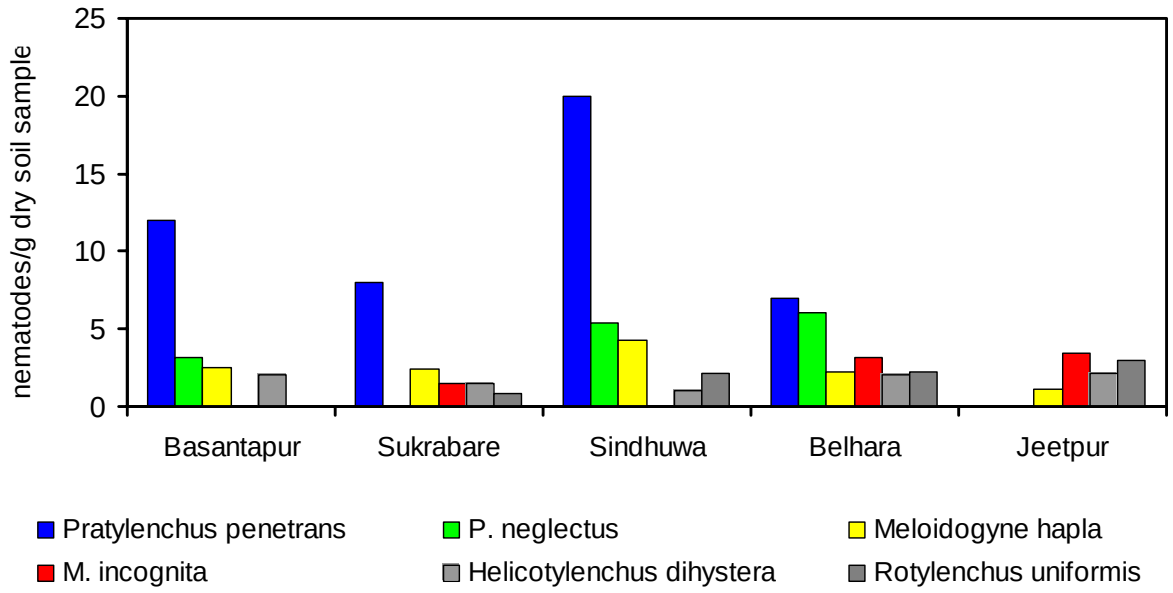

Fig. 4.4 Nematode/g dry soil sample occurs in different location of Tehrathum and Dhankuta district.

4.2 Classification of identified species

4.2.1 *Classification of identified fungi*

The main framework used in this study is based on the classification scheme according to Alexopoulos and Mims (1993).

Division: Mastigomycota
Sub division: Diplomastigomycotina
Class: Oomycetes
Order: Peronosporales
Family: Pythiaceae
Genus: *Pythium* Pringsheim, 1858
Species: ***P. aphanidermatum*** (Edson) Fitzp. 1923
P. ultimum Trow, 1901
P. irregulare Buisman, 1927
P. sulcatum Pratt & Mitchell, 1973

Division: Amastigomycota
Sub division: Deuteromycotina
Form Class: Deuteromycetes
Form sub class: Hyphomycetidae
Form Order: Moniliales
Family: Tuberculariaceae
Genus: *Fusarium* Kew, 1971
Species: ***F. oxysporum*** Schlechtendhal, 1824
F. solani (Martius) Appel et Wollenw.
F. monoliforme Shelden, 1854

Form Order: Agonomycetales
Family: Rhizoctoniaceae
Genus: *Rhizoctonia*
Species: ***R. solani*** Kühn, 1858

Sub division: Ascomycotina
Class: Ascomycetes
Sub class: Hymenoascomycetidae
Order: Helotiales
Family: Sclerotiniaceae
Genus: *Sclerotinia*
Species: ***S. sclerotiorum*** (Lib.) de Bary

4.2.2 *Classification of identified nematodes*

The systematic positions of identified nematodes are according to the framework given by De Ley and Blaxter, (2002) which are as follows:

Phylum: Nematoda Potts, 1932
Class: Chromadorea Inglis, 1983
Subclass: Chromadoria Pearse, 1942
Order: Rhabditida Chitwood, 1933
Suborder: Tylenchina Thorne, 1949
Infraorder: Tylenchomorpha De Ley and Blaxter, 2002
Superfamily: Tylenchoidae Örley, 1880
Family: Hoplolaimidae Filipjev, 1934
Subfamily: Hoplolaiminae Filipjev, 1949
Genus: *Rotylenchus* Filipjev, 1936
Species: ***R. uniformis*** (Thorne, 1949) Loof & Oostenbrink, 1958

Genus: *Helicotylenchus* Steiner, 1945
Species: ***H. dihystera*** (Cobb, 1893) Sher, 1961

Family: Pratylenchidae Thorne, 1949
Subfamily: Pratylenchinae Thorne, 1949
Genus: *Pratylenchus* Filipjev, 1936
Species: ***P. penetrans*** (Cobb, 1917), Filipjev & Schuurmans Stekhoven, 1941
P. neglectus (Rensch, 1924), Filipjev & Schuurmans Stekhoven, 1941

Family: Heteroderidae Filipjev & Schuurmans Stekhoven, 1941
Subfamily: Meloidogyninae Skarbilovich, 1959
Genus: *Meloidogyne* Gölgi, 1892
Species: ***M. hapla*** Chitwood, 1949
M. incognita (Kofoid & White, 1919) Chitwood, 1949

4.3 Identified fungi species

4.3.1 *Pythium aphanidermatum* (Edson) Fitzp. 1923

(Fig. 4.5-4.8)

= *Rheosporangium aphanidermatum* Edson, 1915
= *Pythium aphanidermatum* (Edson) Fitzp., 1923
= *Nematosporangium aphanidermatum* (Edson) Fitzp., 1923
= *Nematosporangium aphanidermatum* (Edson) Jacz., 1931
= *Pythium butleri* Subramaniam, 1919
= *Nematosporangium aphanidermatum* var. hawaiiense Sideris, 1931.

Measurements:

TABLE 4.2 Morphometrics of *Pythium aphanidermatum* (measurements in µm)

HyW	9-10 (10 ± 1)
HySW	19-22 (20.5 ± 1.3)
SpoW	8-22 (14 ± 4)
SpoL	20-50 (35 ± 9)
ZosD	10-13.5 (12 ± 1.2)
ZosL	9-13.5 (11.3 ± 1.2)
OogW	20-25 (22.5 ± 1.5)
AntW	10-14 (12 ± 1.7)
AntL	10-13 (11.5 ± 1.1)
OosD	19-22 (20.5 ± 1.3)
OosWTh	1-2 (1.5 ± 0.3)

Description: Colonies on cornmeal agar with cottony aerial mycelium (Fig. 4.5), on potato-carrot agar with some loose aerial mycelium without a special pattern. The hyphae are hyaline and the mycelium has no cross walls. Main hyphae up to 11 µm wide. Sporangia consisting of terminal complexes of swollen hyphal branches of varying length and up to 22 µm wide. They are lobate (inflated). Zoospores formed at 25-30°C. Encysted zoospores 12 µm diam (Fig. 4.6 & 4.8). Oogonia terminal, globose, smooth, 22.5 µm in diameter, apluerotic oogonium, (oospore doesn't fill the oogonium) (Fig. 4.7 & 4.8). Antheridia mostly intercalary, sometimes terminal, broadly sac-shaped, 10-13 µm long and 10-14 µm wide, 1(-2) per oogonium, monoclinous or diclinous; oospores aplerotic, 20.5 µm in diameter, wall 1-2 µm thick (Fig. 4.7).

Cardinal temperatures: minimum 10°C, optimum 35-40°C, maximum over 40°C. Daily growth rate on potato-carrot agar at 25°C was over 30 mm.

Fig. 4.5 *Pythium aphanidermatum*. Colonies on cornmeal agar with cottony aerial mycelium.

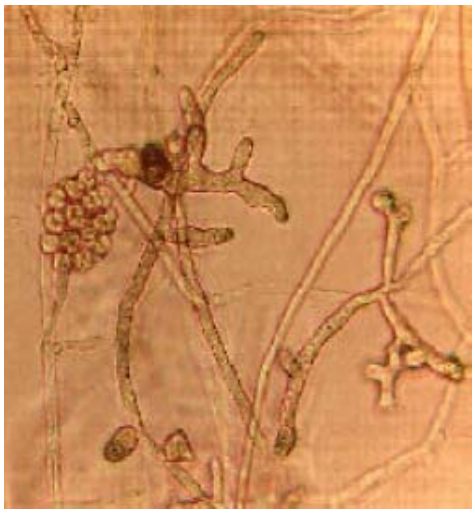

Fig. 4.6 *Pythium aphanidermatum*. Hyphae, zoospores in a vesicle and sporangia.

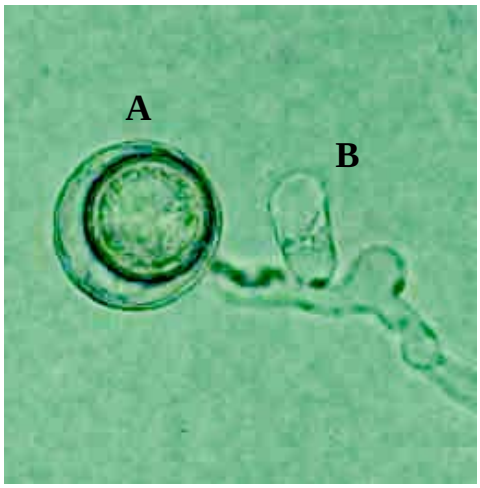

Fig. 4.7 *Pythium aphanidermatum* **A** - Apluerotic oogonium, (oospore doesn't fill the oogonium), **B** - Filamentous sporangium

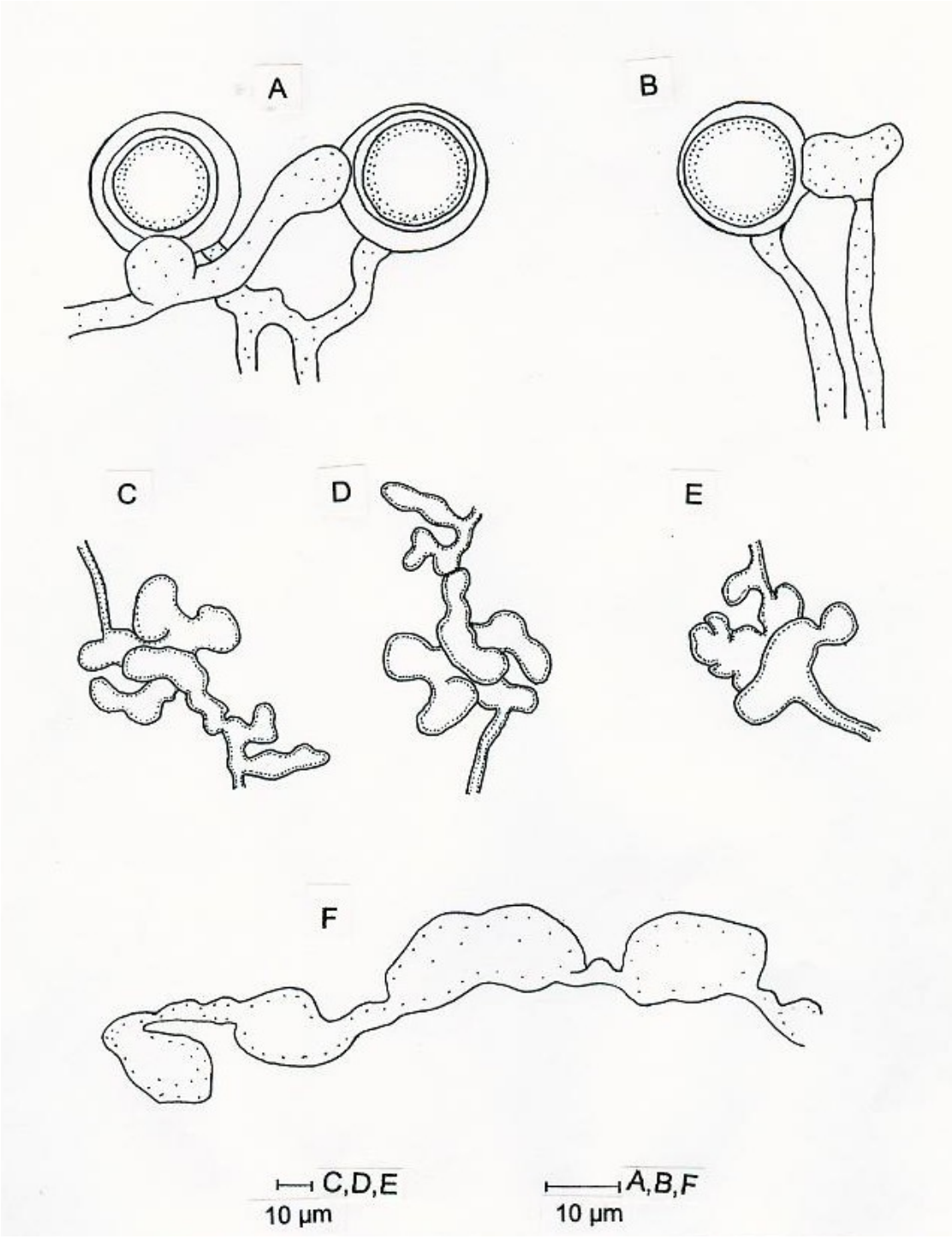

Fig. 4.8 *Pythium aphanidermatum*. **A**, **B** - Oogonia and antheridia; **C**, **D** & **E** - Toruloid sporangia; **F** - Appressoria

4.3.2 *Pythium ultimum* Trow, 1901

(Fig. 4.9, 4.10)

= *Pythium ultimum* Trow, 1901; var. ultimum
= *Pythium debaryanum* sensu de Bary, 1881 (non Hesse 1874)
= *Pythium haplomitrii* Lilienfeld, 1911

Measurements:

TABLE 4.3 Morphometrics of *Pythium ultimum* (measurements in µm)

HyW	8-12 (10 ± 1.5)
HySW	19-22 (20.5 ± 1.3)
SpoW	10-20 (15 ± 4)
SpoL	14-19 (17 ± 2)
ZosD	10-13.5 (12 ± 1.2)
ZosL	9-12.5 (11 ± 1.2)
OogW	19-25 (22.5 ± 1.5)
AntW	6-8.5 (7 ± 1.2)
AntL	9-13 (11 ± 1.2)
OosD	14-20 (18 ± 1.8)
OosWTh	2-3 (2.3 ± 0.3)

Description: Colonies on cornmeal agar forming cottony aerial mycelium, on potato-carrot agar with a radiate pattern. Main hyphae up to 12 µm wide. Sporangia mostly not formed and zoospores very rarely produced through short discharge tubes at 5°C. Hyphal swellings globose, intercalary, sometimes terminal, 23 µm in diameter. Oogonia terminal, sometimes intercalary, globose, smooth-walled, 22.5 µm in diameter; antheridia either 1(-3) per oogonium, small, sac-like, mostly monoclinous originating from immediately below the oogonium, sometimes hypogynous, or 2-3 and then either monoclinous or diclinous and frequently straight. Antheridial stalk never swollen. Oospores single, terminal, aplerotic, globose, 18 µm in diameter, smooth walled often 2 µm or more in thickness (Fig. 4.9 and 4.10). *Cardinal temperatures*: minimum 5°C, optimum 25-30°C, maximum 35°C. Daily growth rate on potato-carrot agar at 25°C was 30 mm.

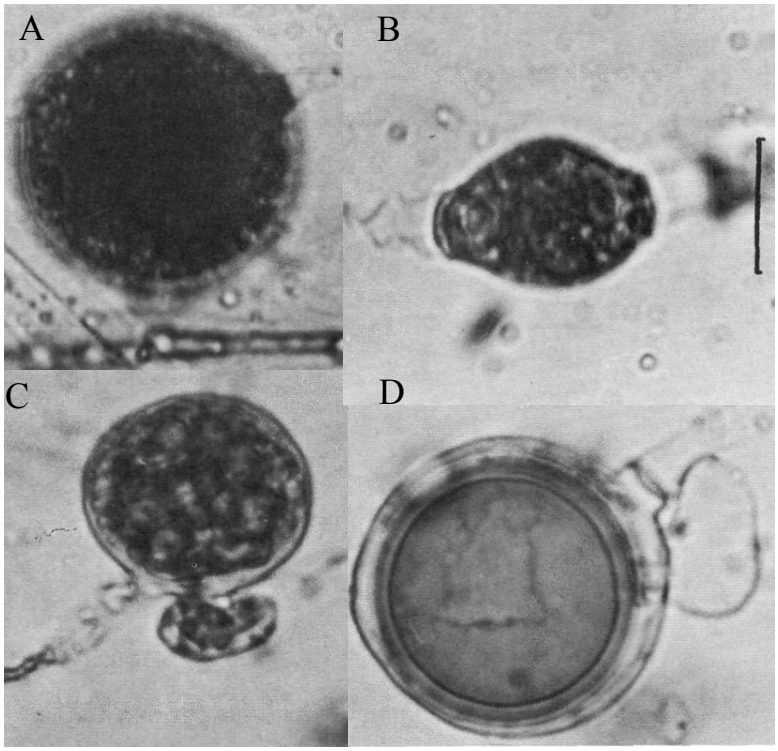

Fig. 4.9 *Pythium ultimum*. **A** - Spherical terminal sporangium, **B** - Intercalary limoniform sporangium, **C** - Young oogonium with typical sessile, monoclinous antheridium and **D** - Aplerotic oospores (Bar = 10µm)

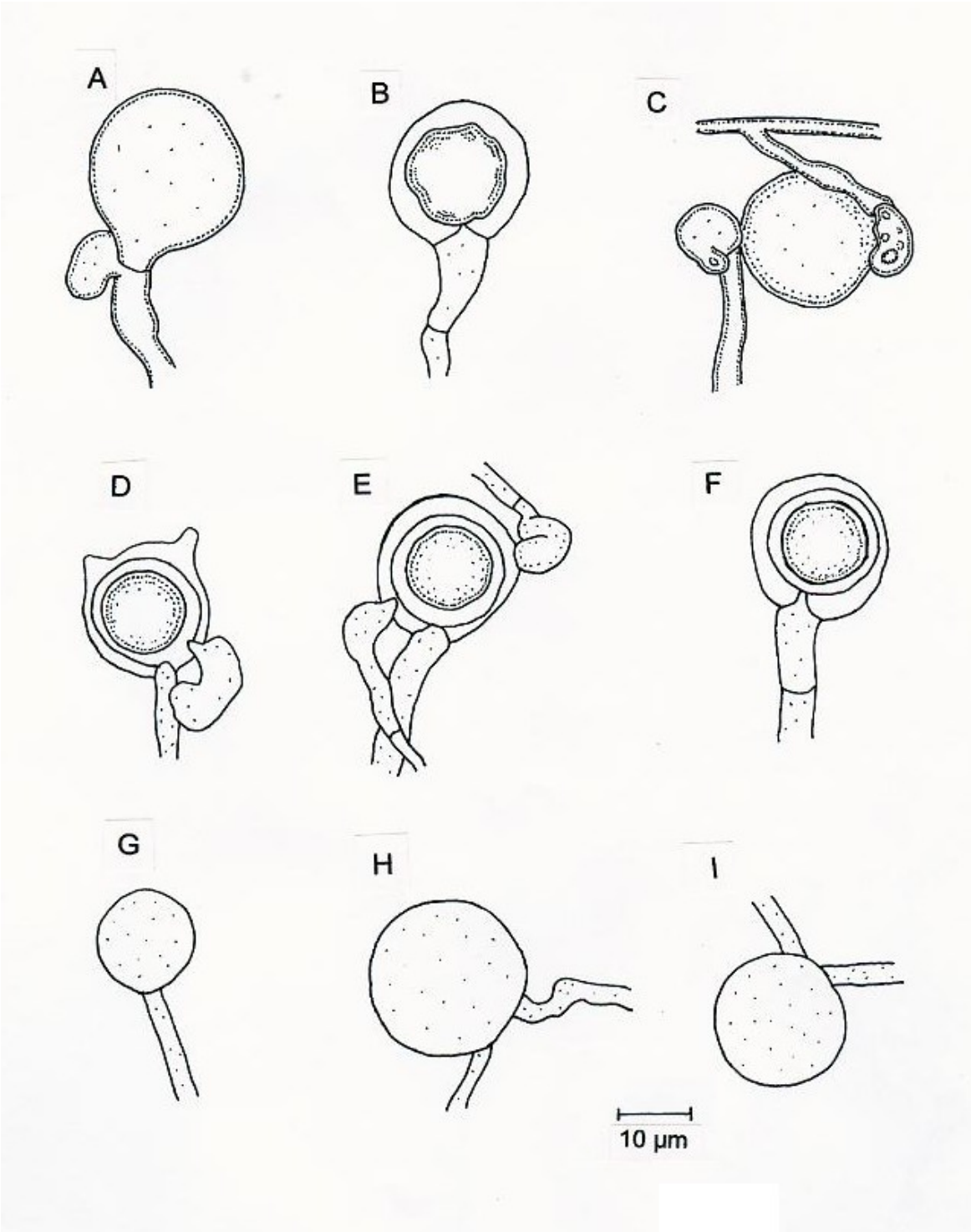

Fig. 4.10 *Pythium ultimum*. **A**, **B** & **C** - Developing stages of oogonia and antheridia, **D**, **E** & **F** - Mature oogonia and antheridia, **B** & **F** - Hypogynous oogonia, **G**, **H** & **I** - Hyphal swellings

4.3.3 *Pythium irregulare* Buisman, 1927

(Fig. 4.11, 4.12)

= *Pythium dactyliferum* Drechsler apud Rands, 1930 (nomen nudum)
= *Pythium fabae* Chenay, 1932
= *Pythium irregulare* var. *hawaiiense* Sideris, 1932
= *Pythium polymorphon* Sideris, 1932

Measurements:

TABLE 4.4 Morphometrics of *Pythium irregulare* (measurements in µm)

HyW	4-6 (5 ± 0.8)
HySW	20-25 (22 ± 2)
SpoW	10-20 (16 ± 3)
SpoL	10-19 (15 ± 3.1)
ZosD	7-8 (7.5 ± 0.3)
ZosL	7-8.2 (7.6 ± 0.4)
OogW	16-23 (19 ± 3)
AntW	4-5 (4.5 ± 0.3)
AntL	10-15 (13 ± 2)
OosD	15-19 (16 ± 3)
OosWTh	1-1.5 (1.2 ± 0.2)

Description: Colonies on cornmeal agar forming a moderate amount of aerial mycelium, on potato-carrot agar some aerial mycelium and a radiate pattern. Main hyphae up to 6 µm wide. Sporangia globose, 10-20 µm in diameter, terminal and intercalary, rarely produced; encysted zoospores about 8 µm diam. Hyphal swellings globose, obovate, limoniform or of irregular shape, terminal and intercalary, up to 25 µm diam. Oogonia globose to irregular, intercalary, sometimes terminal, 19 µm in diameter, smooth or with a varying number of blunt conical or finger-like projections of variable length, mostly 0-5 per oogonium. Antheridia 1-2 per oogonium, monoclinous, mostly stalked and originating at some distance from the oogonium, occasionally sessile, hypogynous, sometimes diclinous; antheridial stalks sometimes branched; antheridial cells 10-15 x 4-5 µm, making apical contact with the oogonium. Oospores mostly aplerotic, occasionally plerotic, 16 µm in diameter, wall mostly 1-1.5 µm thick (Fig. 4.11 and

4.12). *Cardinal temperatures*: minimum 1°C, optimum 30°C, maximum 35°C. Daily growth rate on cornmeal agar at 25°C was 25 mm.

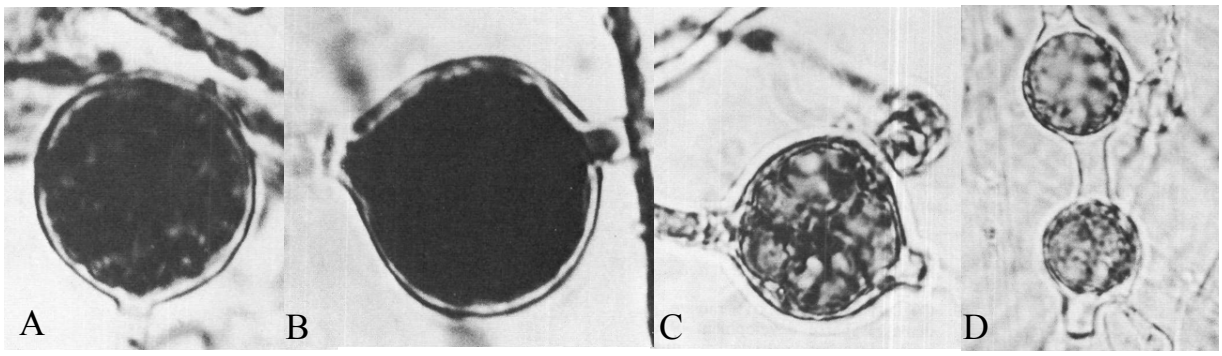

Fig. 4.11 *Pythium irregulare*. **A** - Terminal, **B** - Intercalary, **C** - Intercalary oogonium with monoclinous antheridium and **D** - Oogonia in chain in fertile hyphae

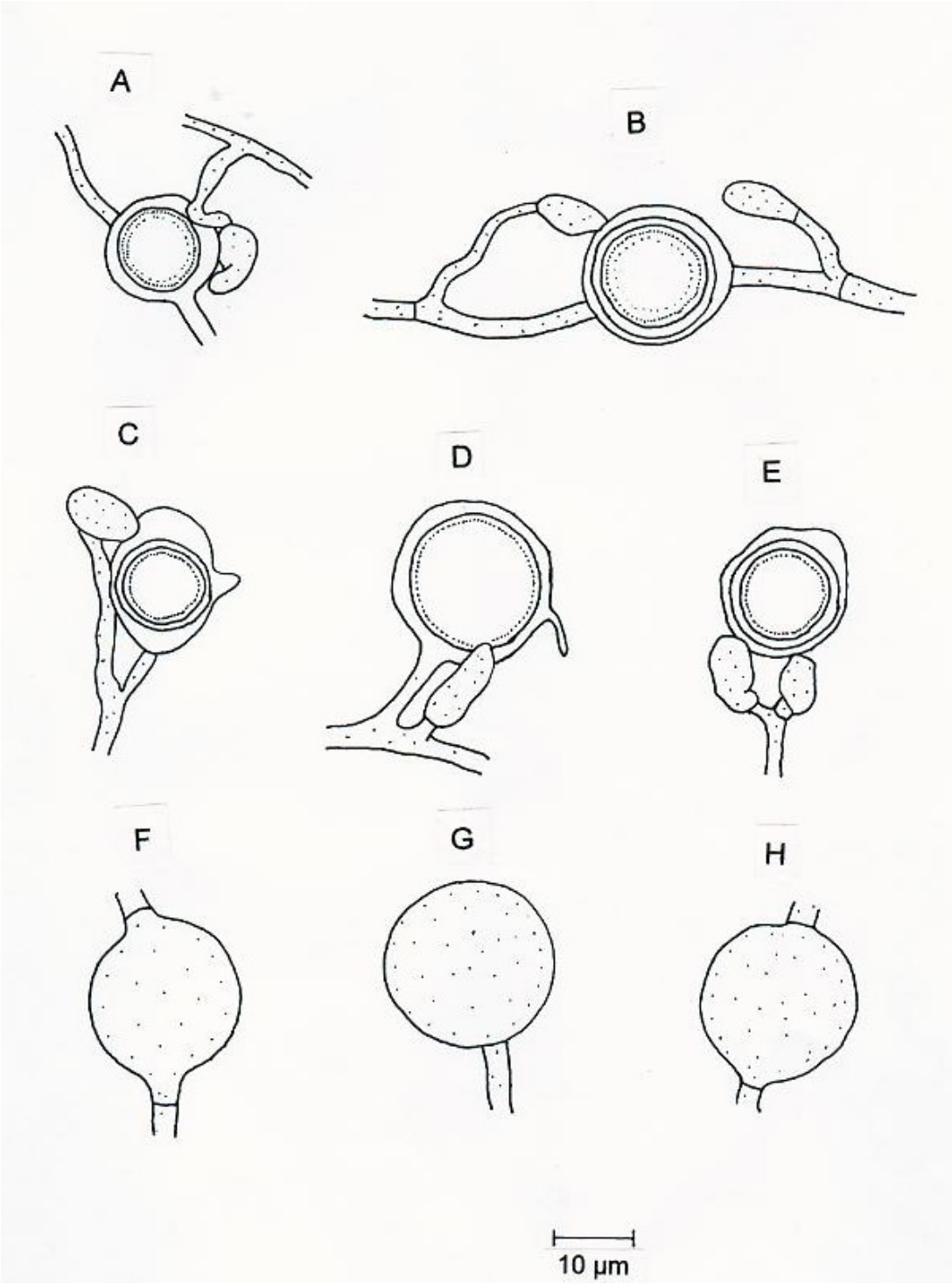

Fig. 4.12 *Pythium irregulare*. **A**, **B**, **C**, **D** & **E** - Oogonia and antheridia, **F**, **G** & **H** - Hyphal swellings

4.3.4 *Pythium sulcatum* Pratt & Mitchell, 1973

(Fig. 4.13)

Measurements:

TABLE 4.5 Morphometrics of *Pythium sulcatum* (measurements in µm)

HyW	4-7 (6 ± 1)
HySW	22-26 (24.5 ± 1.3)
SpoW	4-6.6 (5.1 ± 1)
SpoL	
ZosD	9-13 (11.2 ± 1.5)
ZosL	10-13 (11.3 ± 0.5)
OogW	15-20 (17.5 ± 1.8)
OosD	13-16 (14.5 ± 1.3)
OosWTh	1-1.5 (1.2 ± 0.3)

Description: Colonies on cornmeal agar submerged, on potato-carrot agar with a radiate pattern. Main hyphae up to 7 µm wide. Appressoria sausage-shaped, often catenulate. Hyphal swellings (sub)globose, elongate, obpyriform, ovate or peanut shaped, up to 45 x 26 µm, sometimes formed in the oogonial stalk at varying distances below the oogonium. Sporangia filamentous not differentiated from the vegetative hyphae. Zoospores formed at 20°C. Encysted zoospores 9-13 µm in diameter. Oogonia terminal or intercalary, (sub)globose, smooth, 15-20 µm in diameter. Antheridia 1-3 per oogonium, monoclinous and diclinous; stalks often branched; antheridial cells very variable in size, often large, longitudinally appressed to and encircling the oogonium, having marked or slight folds and furrows, sometimes smaller and clavate or crook-necked. Oospores aplerotic, 13-16 µm in diameter, wall 1-1.5 µm thick (Fig 4.13). *Cardinal temperatures*: minimum 2-3°C, optimum 20-28°C, maximum 36-37°C. Daily growth rate on potato-carrot agar at 25°C was 13-14 mm.

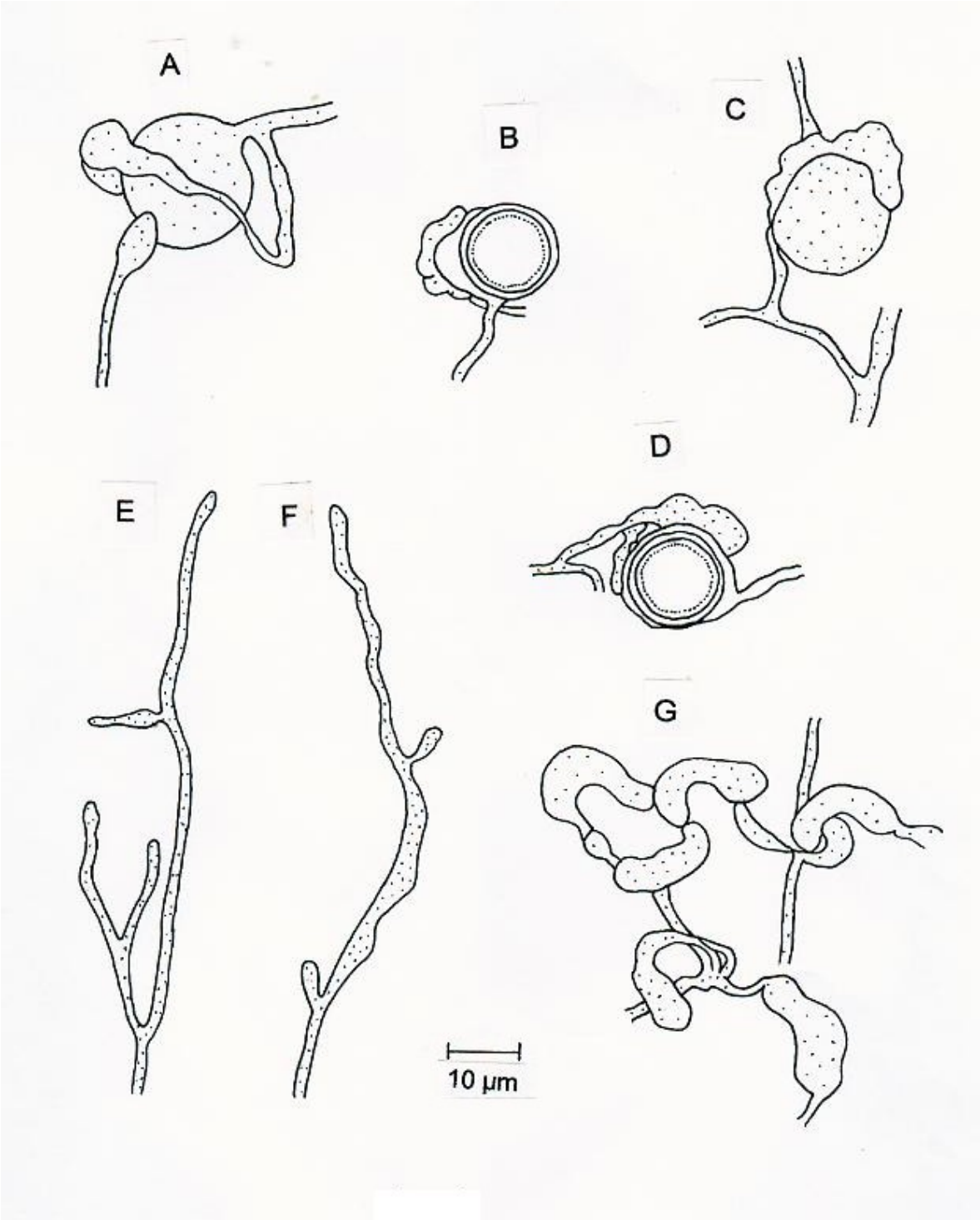

Fig. 4.13 *Pythium sulcatum*. **A**, **B**, **C** & **F** - Oogonia and antheridia, **D** & **E** - Filamentous sporangia, **G** - Appressoria

4.3.5 *Fusarium oxysporum* Schlechtendahl, 1824

(Fig. 4.14, 4.15)

Measurements:

TABLE 4.6 Morphometrics of *Fusarium oxysporum* (measurements in µm)

MicD	2.7-4 (3.4 ± 0.4)
MicL (one celled)	9-13 (11 ± 2)
MicL (two celled)	18-22 (20 ± 1.8)
MacD	3-4.5 (3.8 ± 0.4)
MacL	25-55 (42 ± 11)
ChlD	10-11 (10.4 ± 0.4)

Description: In solid media culture, such as potato dextrose agar (PDA), the colonies of *F. oxysporum* was first appears white and then pigmented with a reddish purple colour and surmounted by a pinkish white aerial mycelium. In case of abundant sporodochia, the culture appeared cream or orange in colour. *F. oxysporum* produces three types of asexual spores: microconidia, macroconidia and chlamydospores. Microconidia one or two celled and most abundantly and frequently produced by the fungus under all conditions, usually comma or ellipsoidal in shape, measured 9-13 µm in length for one celled and 18-22 µm in length for two celled and produced singly. Monophialids produced in aerial mycelium are short and plump. Macroconidia are three to five celled, measured 25-55 µm in length, gradually pointed and generally straight or sometime curved toward the ends. Chlamydospores round, 10-11 µm in diameter, thick-walled spores, produced either terminally or intercalary on older mycelium or in macroconidia. They are found singly rarely in pair (Fig. 4.14 and 4.15). These spores are either one or two celled. Foot cell distinct. Colony diameter after 10 days on PDA was greater than 7 cm.

Fig. 4.14 *Fusarium oxysporum*. Conidia in a tease preparation from a colony. Potato glucose agar, phase contrast microscopy, 630X.

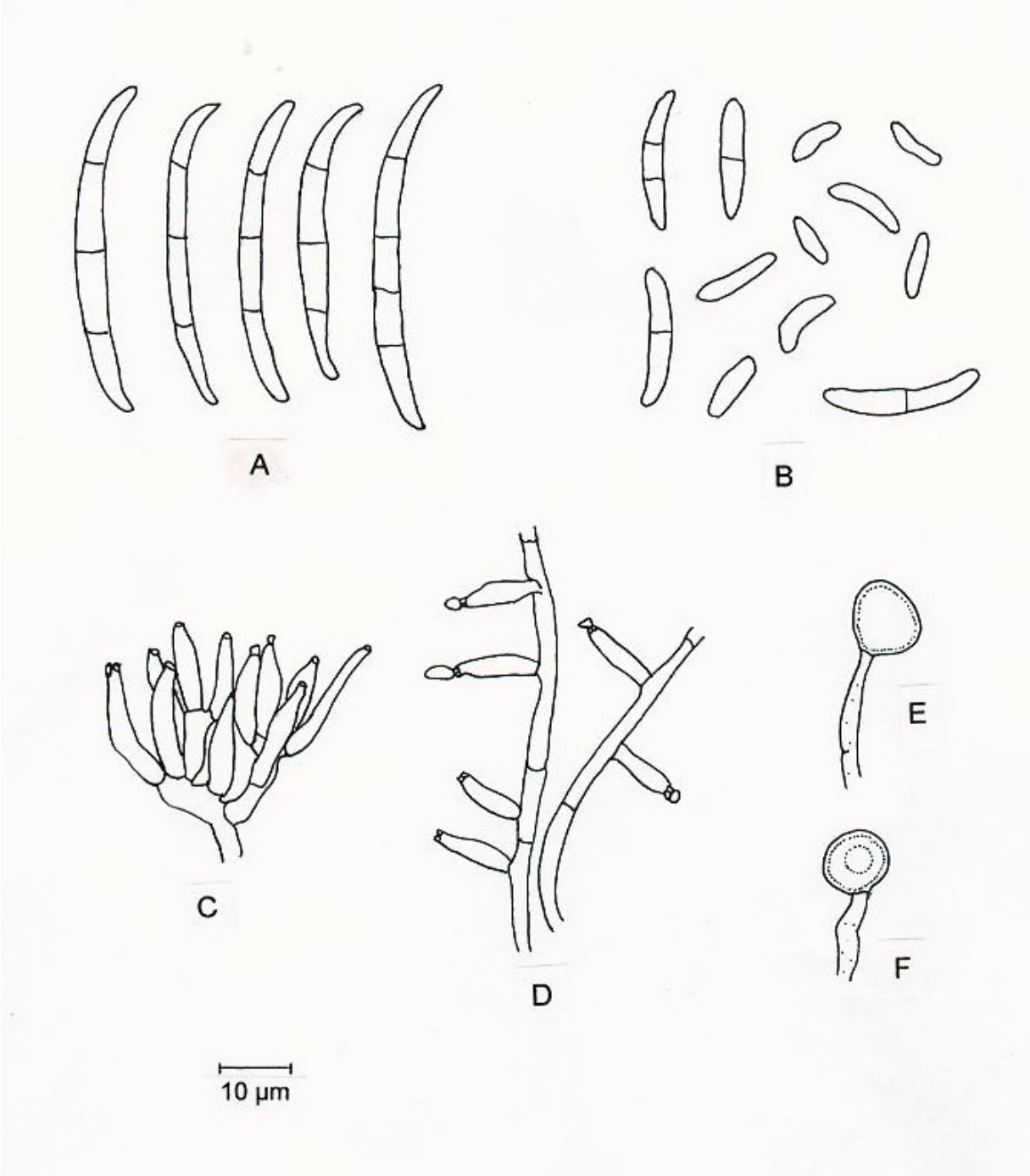

Fig. 4.15 *Fusarium oxysporum*. **A** -Macroconidia, **B** - Microconidia, **C** – Sporodochia, **D** - monophialides, **E** & **F** - Chlamydospores

4.3.6 *Fusarium solani* (Martius) Appel et Wollenw.

(Fig. 4.16, 4.17)

Measurements:

TABLE 4.7 Morphometrics of *Fusarium solani* (measurements in µm)

MicD	2-5 (4 ± 1)
MicL	8-16 (12 ± 3)
MacD	4-6 (5 ± 1)
MacL	35-65 (50 ± 12)
ChlD	10-12 (11 ± 1)

Description: In solid media such as potato dextrose agar (PDA), the colonies of *Fusarium solani* are woolly to cottony with cream to white aerial mycelium and a cream reverse. Sporodochia form and usually moist and cream-colored. Sporodochia sometime occasionally blue-green or blue, but never orange. Hyphae septate and hyaline. Conidiophores simple (non-branched) or branched monophialides (phialides with a single opening). Microconidia borne from long, slender monophialides in the aerial mycelium, one to three-celled, 2-5 x 8-16 µm long, and occur in false heads only (in clusters of conidia at the tip of the phialide) (Fig. 4.16 A). Macroconidia moderately curved, stout, thick-walled, usually 3-5 septate, measure 4-6 x up to 65 µm long, often with rather blunt basal and apical cells and borne on short conidiophores that soon form sporodochia (Fig. 4.16 B). Chlamydospores present (sometimes profuse) and occur both singly and in pairs (Fig. 4.17). Rapid growth, colony diameter after 10 days on PDA was greater than 7 cm.

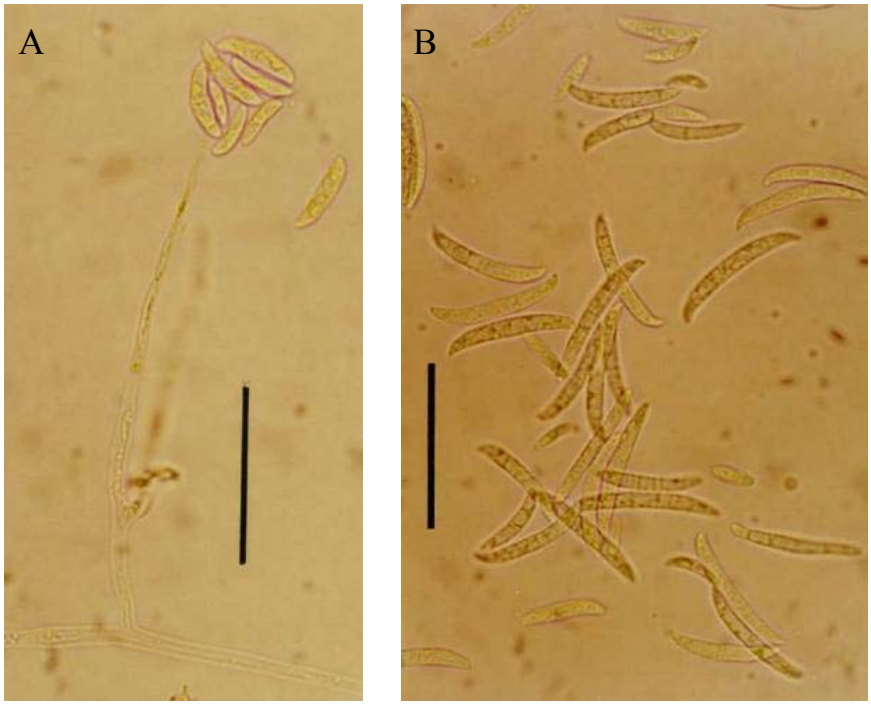

Fig. 4.16 *Fusarium solani*. **A** - Monophialide, forming microconidia (bar 40 µm). **B** - Macroconidia (bar 40 µm)

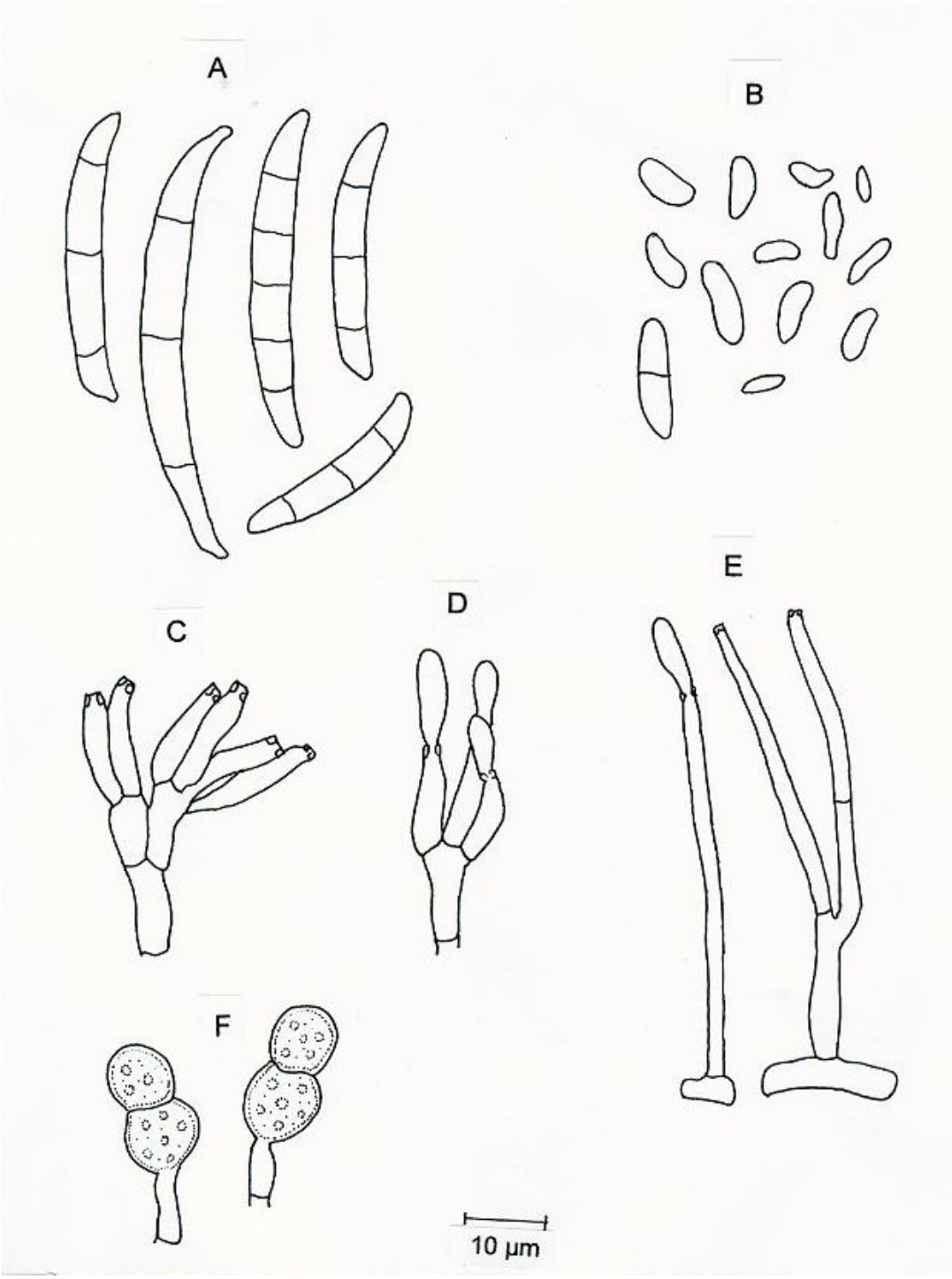

Fig. 4.17 *Fusarium solani*. **A** - Macroconidia, **B** - Microconidia, **C** & **D** - Sporodochia, **E** - monophialides, **F** - Chlamydospores

4.3.7 *Fusarium moniliforme* Sheldon, 1904

(Fig. 4.18)

= *Fusarium verticilloides* (Sacc.) Nirenberg (1976)

Measurements:

TABLE 4.8 Morphometrics of *Fusarium moniliformae* (measurements in µm)

MicD	2.5-3.2 (2.8 ± 0.3)
MicL	7-10 (8.4 ± 1.2)
MacD	2.7-3.6 (3.1 ± 0.3)
MacL	31-58 (44 ± 11)

Description: In solid media such as potato dextrose agar (PDA), the colonies of *Fusarium moniliforme* are initially white becoming tinged with lavender with a colourless to dark purple reverse. The descriptions here are based upon growth at 25°C with on/off fluorescent light cycles of approximately 12 hours each. Sporodochia, when formed, are cream to orange, however are generally sparse on PDA. Dark blue sporodochia may be present. Hyphae are septate and hyaline. Conidiophores are medium length simple or branched. Conidiogenous cells are monophialides. Microconidia produced from monophialides in the aerial mycelium, abundant, 0 to 1-septate, oval to clavate, truncated at base, measure 7-10 x 2.5-3.2 µm and occur in both false heads (a collection of conidia at the tip of the phialide) and in chains. Macroconidia are sparse, narrow and very slightly sickle-shaped to nearly straight, i.e., 'string bean-like', 5-septate, measuring 31-58 x 2.7-3.6 µm (Fig. 4.18). Chlamydoconidia are absent. Rapid growth. colony diameter after 10 days on PDA was greater than 7 cm.

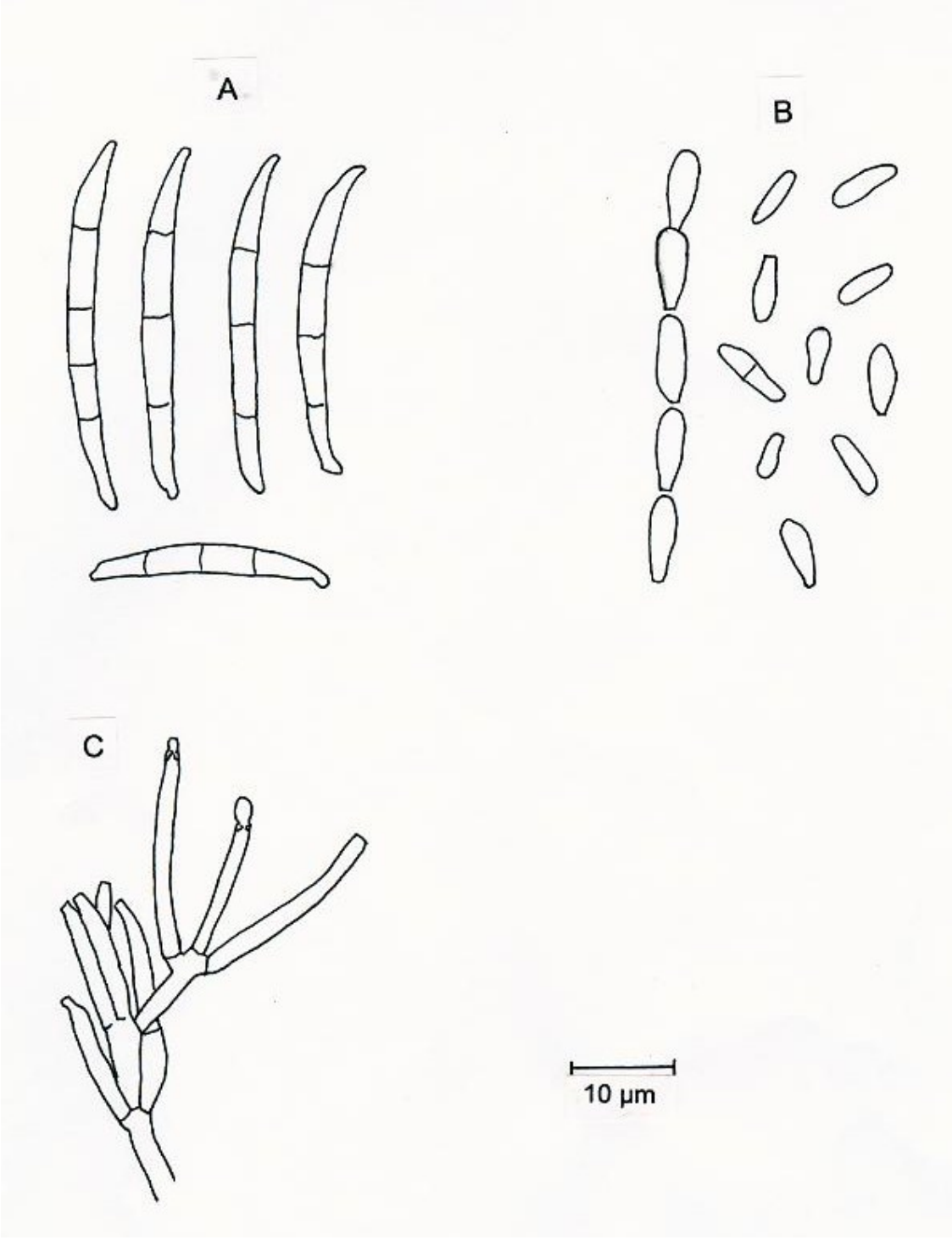

Fig. 4.18 *Fusarium moniliforme*. **A** - Macroconidia, **B** - Microconidia, Chlamydospores, **C** – Sporodochia with monophialides

4.3.8 *Rhizoctonia solani* Kühn, 1858

(Fig. 4.19, 4.20)

Description: The vegetative myceliums of *R. solani* are colourless when young but become pale to dark brown coloured as they grow and mature. The mycelium partitioned into individual cells by a septum containing a dough-nut shaped pore, branched near the septum, nearly right angle to the hyphae, hyphae constrict near the point of origin, the main runner hyphae usually wider than 7 µm. The hyphal cells usually possess more than three nuclei. The cellular nuclear number (CNN) to the tips in young hyphae greater than two. Sclerotia irregular shaped, light to dark brown, not differentiated into rind and medula. No clamp connections (Fig. 4.19 and 4.20).

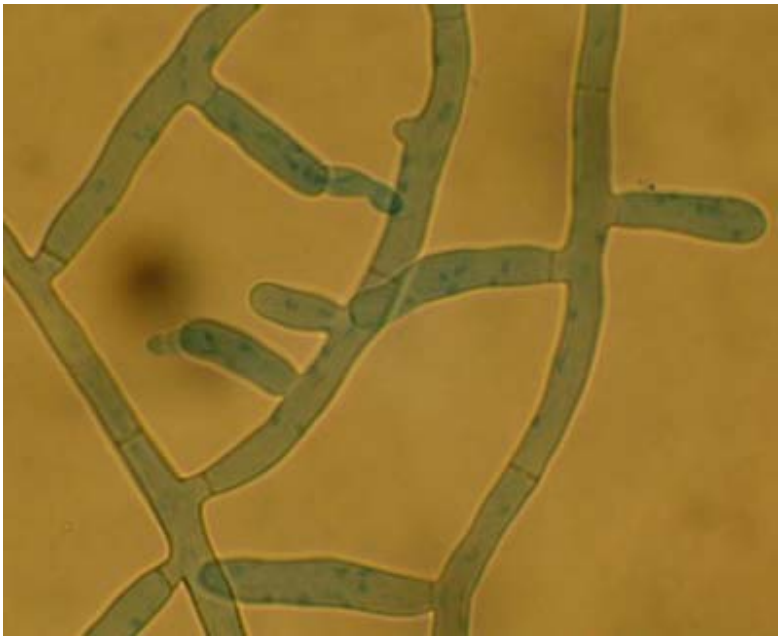

Fig. 4.19 *Rhizoctonia solani*. Hyphae showing septa and nuclei

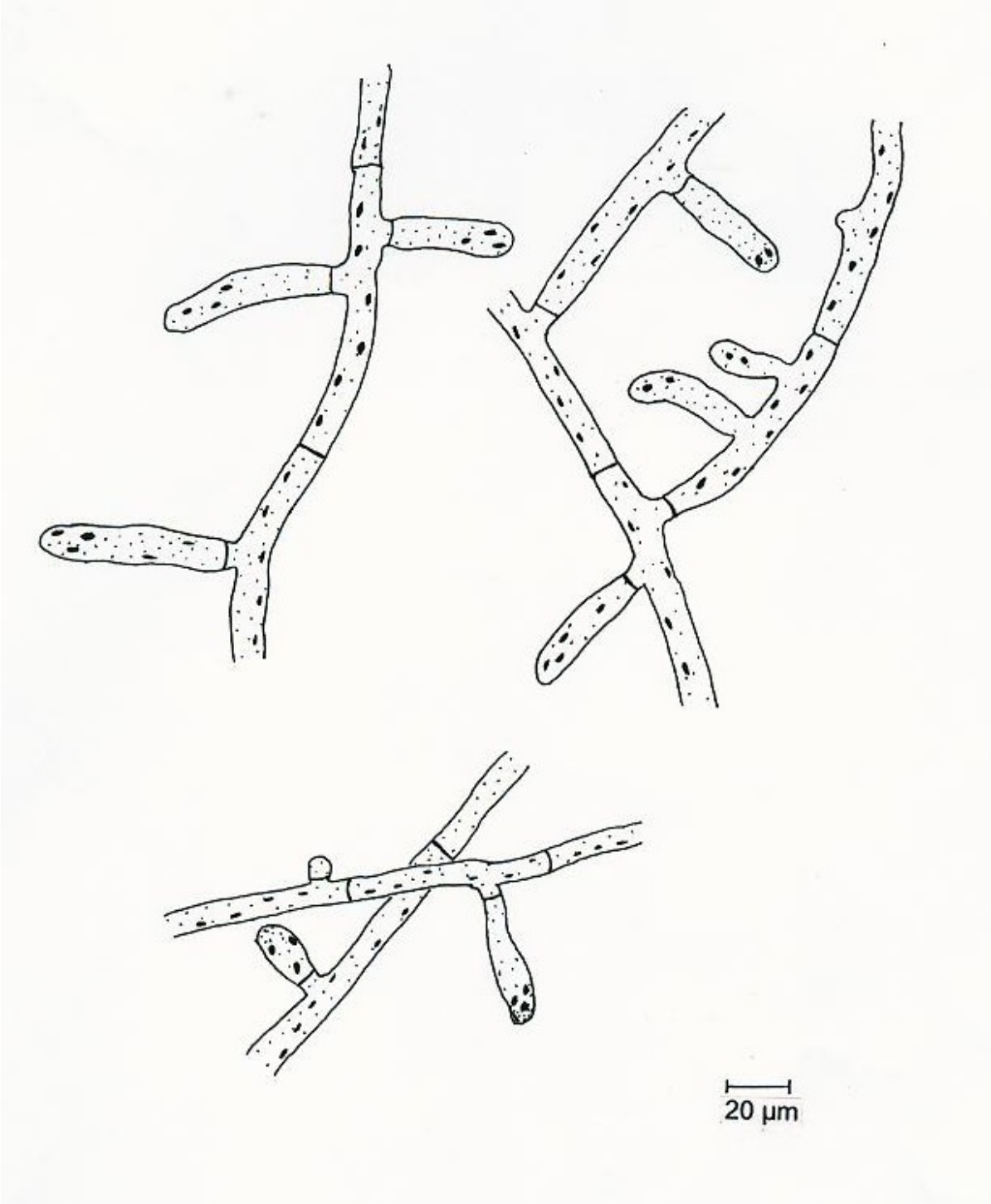

Fig. 4.20 *Rhizoctonia solani*. Mycelium showing matured hyphae with cellular nuclei

4.3.9 *Sclerotinia sclerotiorum* (Lib.) de Bary

(Fig. 4.21, 4.22)

Description: Mycelium of *S. sclerotiorum* gives rise to sclerotia that later germinate to produce apothecia. After apothecia have formed, they mature to produce asci containing ascospores. These ascospores will discharge from the asci, land on host tissue, and germinate to form hyphae. The hyphae will infect lower stems and roots eventually invading plant tissues, which will result in collapse. Mycelium will continue invading tissues while spreading throughout the rest of the plant and eventually form sclerotia (Fig. 4.21 and 4.22).

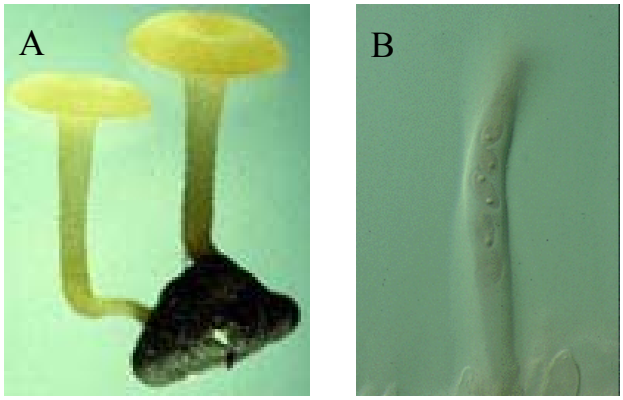

Fig. 4.21 *Sclerotinia sclerotiorum*. **A** - Apothecium producing asci. **B -** Ascus with ascospores

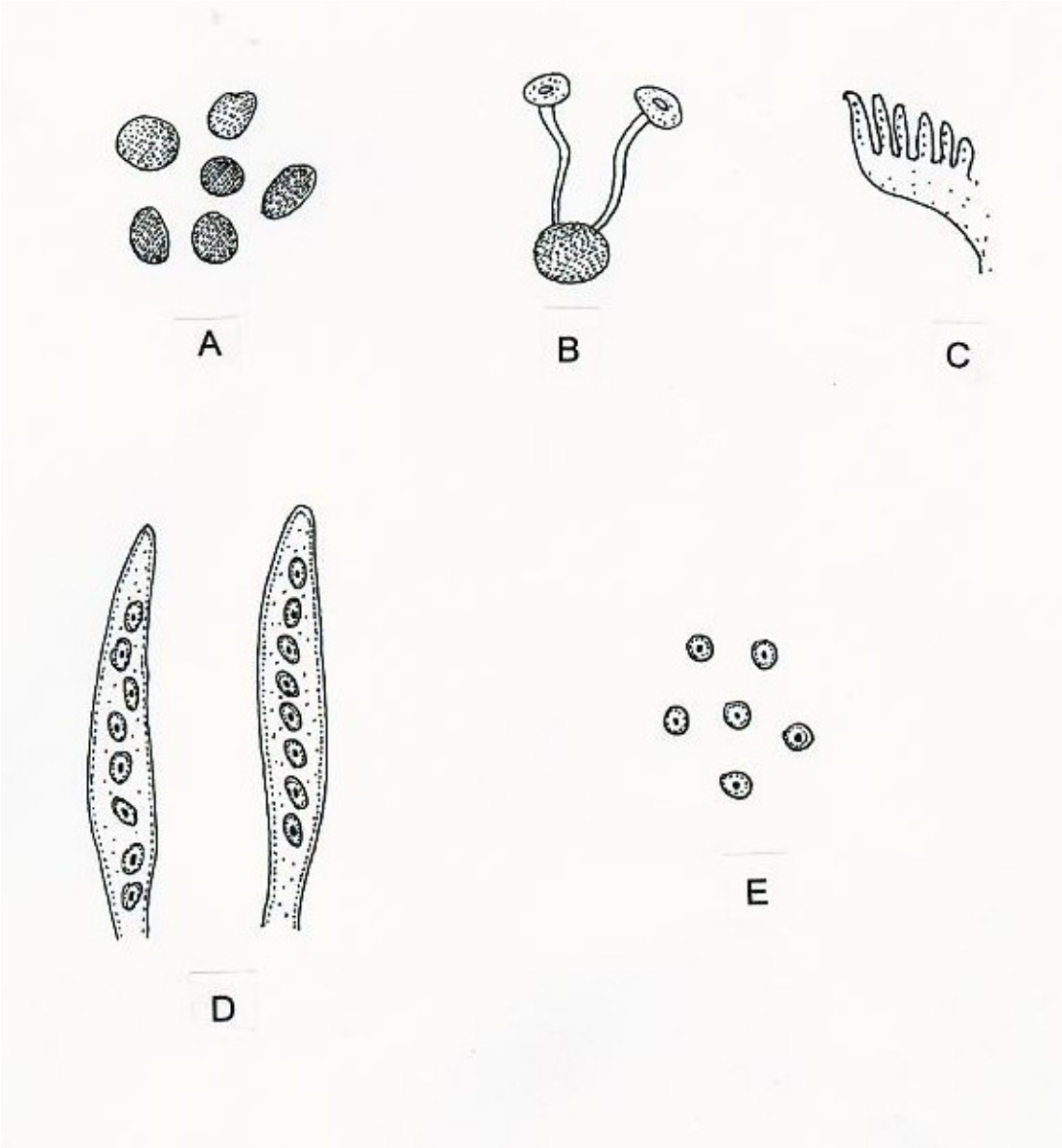

Fig. 4.22 *Sclerotinia sclerotiorum*. **A** – Sclerotium, **B** – Sclerotium germinated into apothecia, **C** – Apothecium produce asci with ascospores, **D** – Ascus with ascospores, **E** – Ascospores

4.4 Identified nematodes species

4.4.1 *Rotylenchus uniformis* (Thorne, 1949) Loof & Oostenbrink, 1958

(Fig. 4.23)

Measurements:

TABLE 4.9 Morphometrics of *Rotylenchus uniformis* (measurements in µm)

N	20 ♀♀	10 ♂♂
L	1112-1870 (1420 ± 240)	
a	28-39 (32 ± 3)	
b	8.1-11.0 (9.2 ± 1.5)	
b’	6.0-8.7 (7.3 ± 1.2)	
c	45-112 (60 ± 22)	
c’	0.5-1.5 (0.7 ± 0.2)	
Body width	39-47 (44 ± 4)	
V	49-59 (55 ± 3.5)	
Stylet	39-50 (44 ± 4.5)	40-44 (42 ± 1)
Pharynx	137-181 (153 ± 17)	
Pharyngeal gland lobe	33-63 (44 ± 14)	
E. pore	137-189 (161 ± 25)	
Tail	16-35 (23 ± 8)	
Abw	23-32 (29 ± 3)	
Spicules		34-40 (37 ± 2)
Gub.		17-21 (19 ± 1.4)

Description: *Female*: Body loose spiral and long. Lateral field regularly areolated in pharynx region and then sparsely areolated on entire body. Head spherical, offset by constriction, annuli 5-7, irregularly divided by longitudinal striae. Cephalic framework strongly refractive. Stylet robust, cone slightly longer than shaft, knobs rounded to anteriorly flattened, sometimes slightly concave, 7-9 µm across. Excretory pore 137-189 (161) µm from anterior end. Pharyngeal lobe extends over intestine for 0.9-1.8 (1.2) body widths. Spermatheca with elongated sperm, epiptygma double. Phasmids 8 annuli anterior to 3 annuli posterior to anus. Tail hemispherical, sometimes curved on dorsal side, with 8-18 (12) annuli (Fig. 4.23).

Male: Body less curved, stylet 40-44 µm long, weaker than in female, spicules 34-40 µm gubernaculums 17-21 µm long.

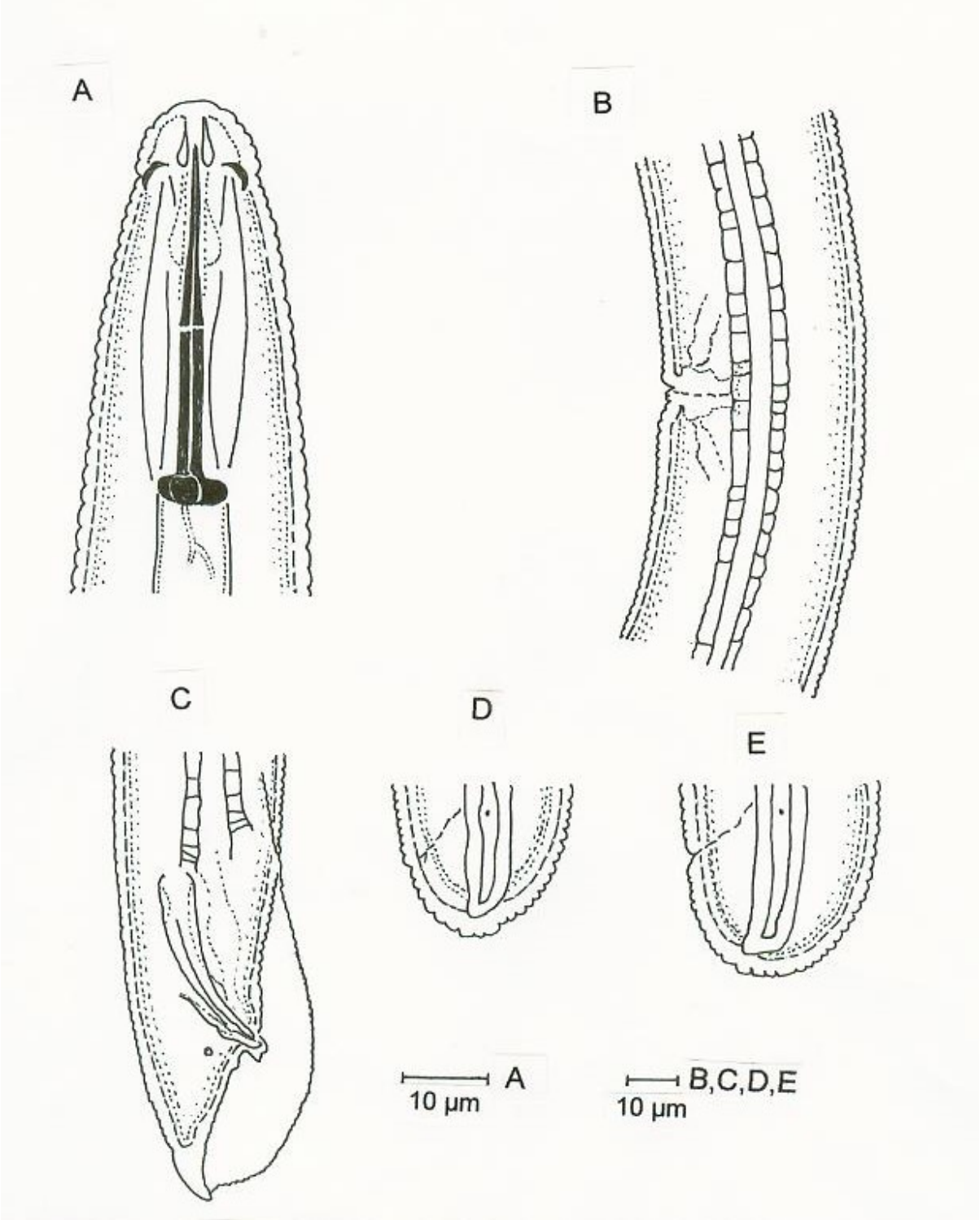

Fig. 4.23 *Rotylenchus uniformis*. **A** – Female head, **B** – Lateral field areolation, **C** – Male tail, **D** & **E** – Female tails.

4.4.2 *Helicotylenchus dihystera* (Cobb, 1893) Sher, 1961

(Fig. 4.24)

(Syn. after Fortuner, 1987)
= *Tylenchus olaae* Cobb, 1906
= *T. spiralis* Cassidy, 1930
= *Aphelenchus dubius peruensis* Steiner, 1920
= *Helicotylenchus nannus* Steiner, 1945
= *H. crenatus* Das, 1960
= *H. flatus* Roman, 1965
= *H. rotundicauda* Sher, 1966
= *H. punicae* Swarup & Sethi, 1968
= *H. glissus* Thorne & Malek, 1968
= *H. dihysteroides* Siddiqi, 1972
= *H. teleductus* Anderson, 1974

Measurements:

TABLE 4.10 Morphometrics of *Helicotylenchus dihystera* (measurements in µm)

N	19 ♀♀
L	578-698 (630 ± 39)
a	25-31 (27.4 ± 1.5)
b	5.3-6.5 (5.9 ± 0.4)
b'	3.9-5.1 (4.5 ± 0.3)
c	40-48 (43 ± 3)
c'	0.9-1.1 (1.1 ± 0.1)
Body width	21-25 (23 ± 2)
V	62-66 (64 ± 1) %
Stylet	24-26 (24 ± 0.6)
M	46-52 (49 ± 2) %
DGO	9-11 (9 ± 1)
O	35-42 (38 ± 3) %
Pharynx	92-129 (107 ± 9)
Pharyngeal gland end	119-161 (142 ± 11)
mb	66-77 (72 ± 4)
MB	60-73 % (67 ± 4)
E. pore	86-122 (103 ± 9)
Tail	12-16 (15 ± 1)
Abw	13-15 (14 ± 1)
Annul.	1.4-1.6 (1.5 ± 0.1)
R Ph	5-9 (7 ± 1)

Description: *Female*: General body posture varying from a loose spiral with nearly straight anterior end to a tight regular spiral of almost two turns). Lip region hemispherical with slightly depressed oral opening, with 4 to 5 more or less distinct annules (Fig. 4.24). Outer margin of labial framework conspicuous. Both anterior and posterior cephalids visible. Stylet with flattened to indented knobs. Hemizonid not visible, excretory pore anterior to or level with pharyngeo-intestinal junction.

Reproductive system didelphic, anterior gonad longer than posterior. Vulva depressed, vagina with epiptygmata (Figs. 4.24, B). Spermatheca conspicuous, offset, empty. Lateral field areolated or not areolated (Fig. 4.24, D & E). Inner incisures fusing near middle of tail. Tail length variable, corresponding to roughly one anal body width, dorsally convex with 7-9 annules and a small ventral projection that is shorter than two body annules.

Male: Not found.

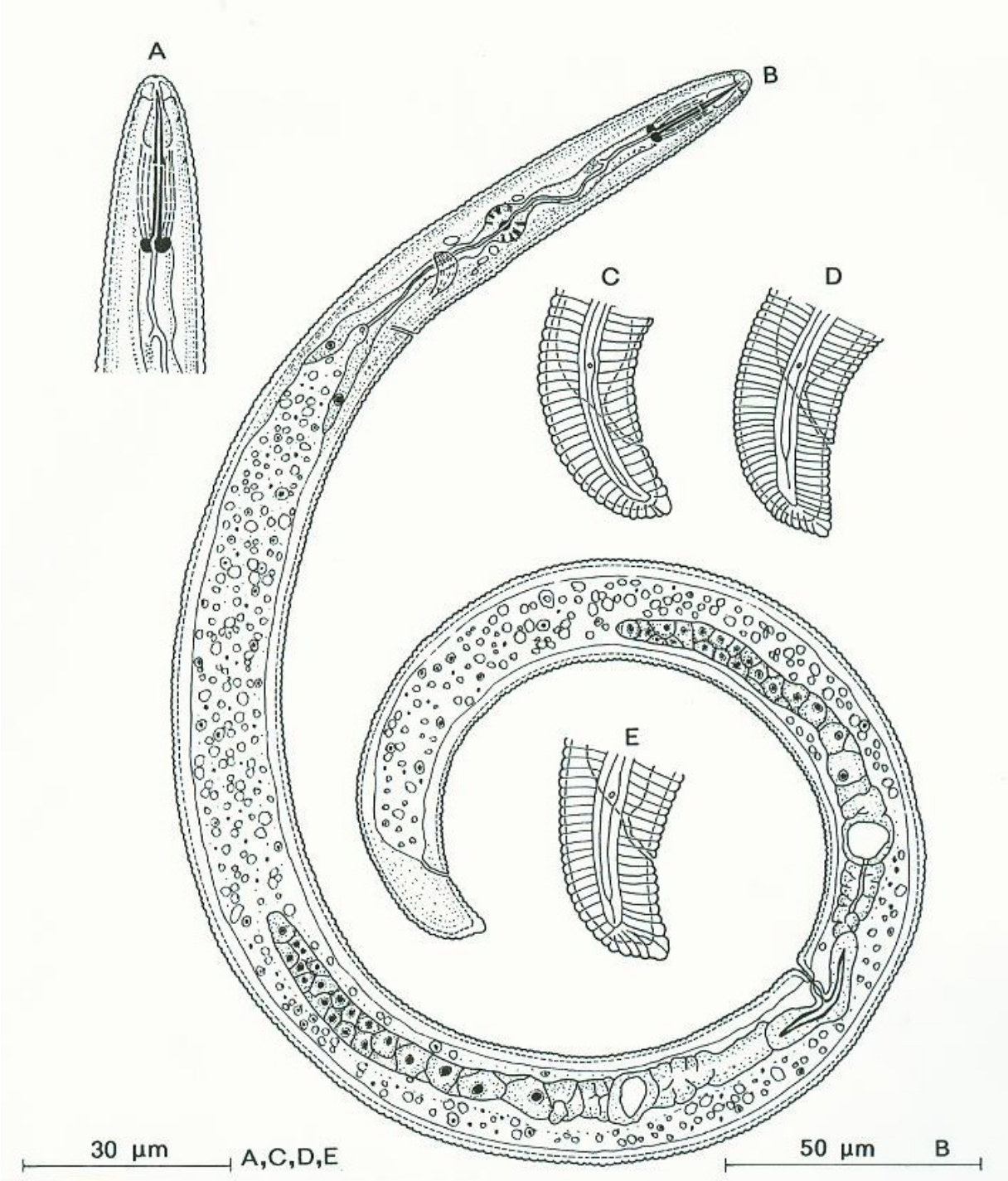

Fig. 4.24 *Helicotylenchus dyhistera*. **A** – Head, **B** – Entire body, **C**, **D** & **E** – Tail variations

4.4.3 *Pratylenchus penetrans* (Cobb, 1917), Filipjev & Schuurmans Stekhoven, 1941

(Fig. 4.25)

= *Tylenchus gulosus* Kuhn, 1890; Fischer, 1894;
= *Tylenchus penetrans* Cobb, 1917;
= *Tylenchus pratensis* Steiner, 1927;
= *Anguillulina pratensis* Goodey, 1932;
= *Pratylenchus pratensis* Filipjev & Schuurmans Stekhoven, 1941

Measurements:

TABLE 4.11 Morphometrics of *Pratylenchus penetrans* (measurements in µm)

N	19 ♀♀	10 ♂♂
L	521-660 (660 ± 24)	
a	22-30 (28 ± 2.1)	
b	5.9-7.7 (6.5 ± 0.6)	
b'	3.3-3.9 (3.7 ± 0.2)	
c	15-22 (18 ± 2.3)	
c'	1.8-2.4 (2.0 ± 0.2)	
Body width	17-22 (19 ± 3)	
V	73-83 (80 ± 3) %	
Stylet	14-16.5 (15.3 ± 0.8)	
DGO	1.1-3.1 (2 ± 0.6)	
Pharynx	48-70 (61 ± 6)	
Pharyngeal gland end	105-139 (114 ± 10)	
Pharyngeal gland lobe	45-69 (54 ± 7)	
E. pore	61-78 (71 ± 6)	
Tail	21-29 (26 ± 3)	
PVUS	16-21 (18.6 ± 1.5)	
Abw	10-14 (12.5 ± 1.3)	
Spicules		12-17 (15.5 ± 2.2)
Gub.		3.6-4.8 (4.0 ± 0.5)

Description: *Female*: *Pratylenchus penetrans* are small nematodes measuring less than 0.9 mm in length. They are moderately slender, almost straight when killed by mild heat. Cuticular annulations are fine and lateral field contain four incisures. Lip region is composed of 3 annules and slightly set off and low. The head is strong and conspicuously slerotized and flattened with rounded edge. The basal knob of stylet is broadly rounded, sometimes cupped

anteriorly. Pharyngeal glands overlap intestine ventrally. Excretory pore is about opposite of pharyngeo-intestine junction with hemizonid occupying about two body annules with ampulla. No sexual dimorphism is present in the anterior part of the body. The vulva is posterior and females are monodelphic with reduced post uterine sac which is undifferentiated and 1 to 1.5 vulval body width long. Spermatheca is spherical or nearly so and may or may not develop well. Tail is rounded, tip smooth or sometimes crenate, with 15-27 annules on ventral surface (Fig. 4.25).

Males: Common. Lateral field has four incisures ending on the bursa enveloping tail tip. Spicules are slender, with distinct manubria and ventrally arcuate shafts. Gubernaculum is simple. Tail tip pointed (Fig. 4.25).

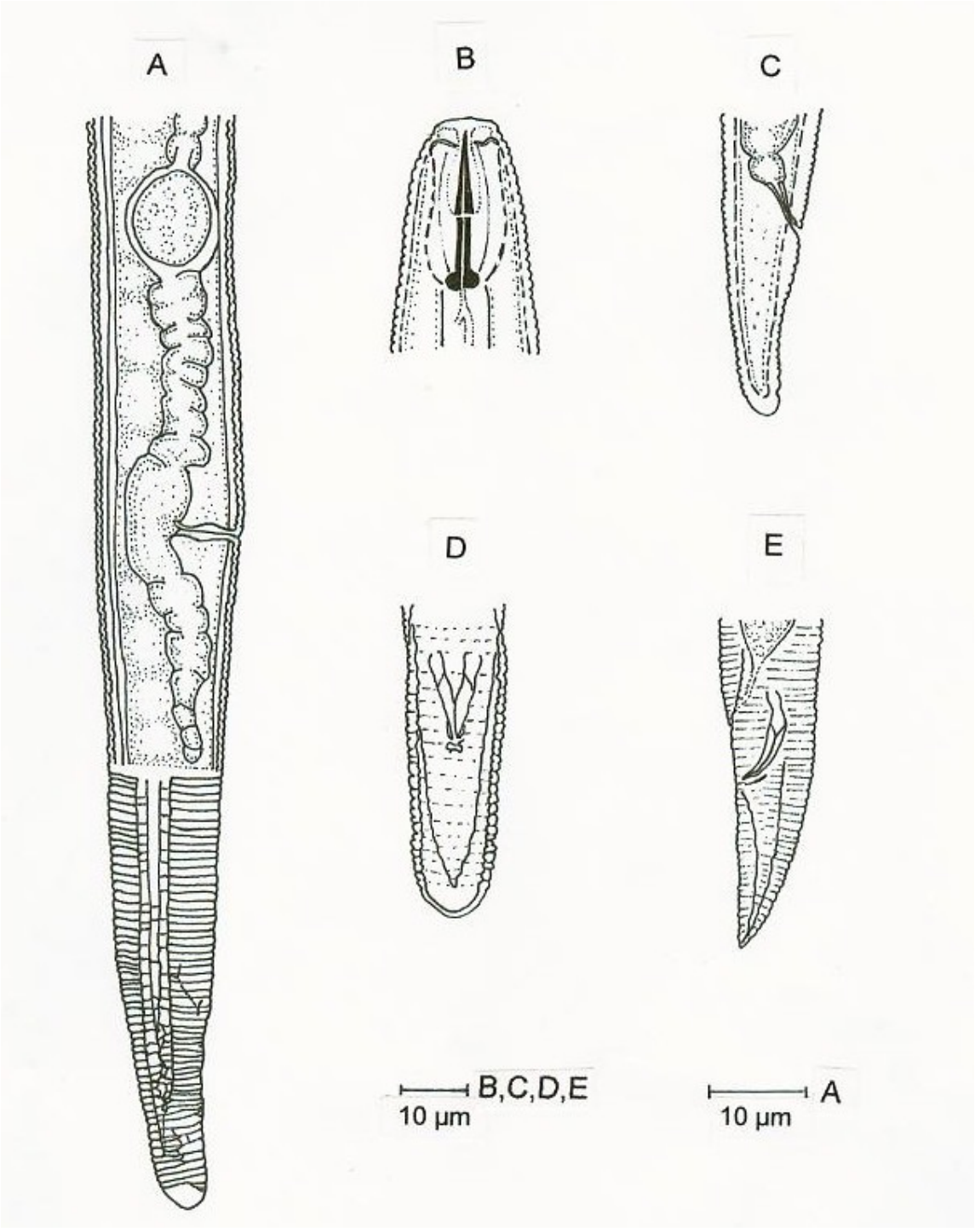

Fig. 4.25 *Pratylenchus penetrans*. **A** – Female vulval region and tail, **B** – Female head, **C** – Female tail tip, **D** – Male tail in ventral view, **E** – Male tail in lateral view

4.4.4 *Pratylenchus neglectus* (Rensch, 1924), Filipjev & Schuurmans Stekhoven, 1941

(Fig. 4.26)

= *Aphelenchus neglectus* Rensch, 1924
= *Aphelenchus neglectus* Goffart, 1927
= *Tylenchus neglectus* Steiner, 1928
= *Anguillulina neglectus* W. Schneider, 1939
= *Pratylenchus minyus* Sher & Allen, 1953
= *Pratylenchus capitatus* Loof, 1978

Measurements:

TABLE 4.12 Morphometrics of *Pratylenchus neglectus* (measurements in µm)

N	12 ♀♀
L	321-588 (460 ± 84)
a	19-31 (24 ± 4)
b	4.9-7.8 (6.1 ± 1)
b'	3.6-5.0 (4.1 ± 0.6)
c	14-27 (20 ± 3)
c'	1.5-2.5 (2.0 ± 0.3)
Body width	17-22 (19 ± 3)
V	75-87 (82 ± 4) %
Stylet	15-19 (16.2 ± 0.8)
DGO	1.1-3.1 (2 ± 0.6)
Pharynx	62-82 (73 ± 6)
Pharyngeal gland end	105-139 (114 ± 10)
Pharyngeal gland lobe	20-42 (31 ± 7)
E. pore	63-81 (75 ± 6)
Tail	16-26 (21 ± 3)
PVUS	12-18 (15 ± 2)
Abw	11-14 (12 ± 1)

Description: *Females*: A parthenogenetic species characterized by great variation in body length and width, tail shape and thickness and early maturity of adults which is sometimes completed during the final ecdysis. Feeding, mature females extracted from roots are often markedly more robust than females obtained from the rhizosphere, which become more linear when relaxed. Head with 2 annules of about equal size, the apical one comprising the lips. The

dorsal and ventral submedian lips are fused, forming a large 'head cap', while the lateral lips are delineated and the amphid apertures are at an oblique angle. Spear knobs 4 to 6 µm across, typically indented on anterior surfaces. Dorsal lateraly over intestine, the subventral gland nuclei at end of lobes, not in tandem. Excretory pore 63-81 (75) µm from the head end. Hemizonid immediately anterior to excretory pore, extending over 2-3 body annules. Lateral field with 4 incisures, but median zone is often marked by 1 or 2 longitudinal or several oblique striae. Female monodelphic, prodelphic, ovary outstretched with oocytes in tandem and occasionally extending to base of oesophagus. Intra-uterine eggs in older adults may be segmented or contain early 1st stage larva. Post-uterine branch less than or equal to body width, 12-18 µm long and is undifferentiated. Tail variable in shape usually conoid with little curvature of ventral surface and usually with 15-20 annules. Tail terminus without annulation, usually rounded, but may be obliquely truncate or slightly digitate. Phasmids in posterior half of tail (Fig. 4.26).

Males: Never found.

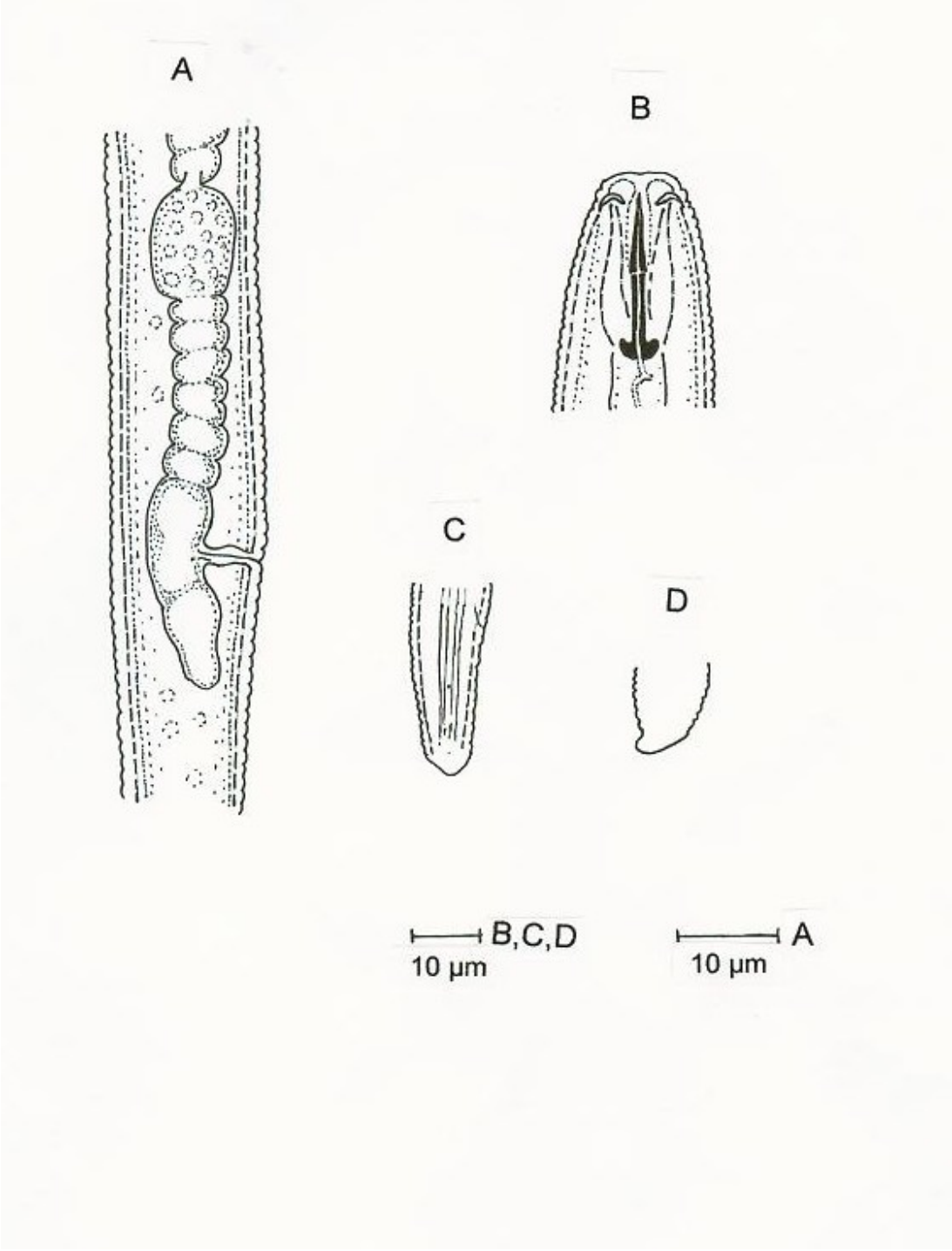

Fig. 4.26 *Pratylenchus neglectus*. **A** – Female vulval region, **B** – Female head, **C** & **D** – Female tail variations

4.4.5 *Meloidogyne hapla* Chitwood, 1949

(Fig. 4.27, 4.29)

Measurements:

TABLE 4.13 Morphometrics of *Meloidogyne hapla* (measurements in µm)

N	15 ♀♀	10 ♂♂	20 J_2
L	421-788 (600 ± 124)	780-1230 (1000 ± 214)	312-330 (320 ± 9)
W	317-540 (420 ± 94)		
a		33-45 (40 ± 3)	20-26 (23 ± 2)
b”		13-19 (15 ± 3)	7.1-9.8 (8.1 ± 1.1)
c		73-197 (125 ± 40)	7-10 (8.5 ± 1.2)
c'			3.5-4.6 (4.1 ± 0.3)
Stylet	10-14 (12 ± 1.5)	19-21 (20 ± 0.3)	7.5-10 (8.8 ± 0.6)
SbW	2-3 (2 ± 0.7)	2.5-4.8 (3.4 ± 1.2)	
DGO	4-6 (5 ± 0.7)	2.5-3.2 (2.9 ± 0.3)	
MbL	31-42 (35 ± 5)	16-25 (20 ± 3)	
MbW	27-37 (32 ± 4)	7-13 (11 ± 2)	
MbVL	10-13 (11 ± 1)	3.6-7.3 (6.1 ± 1)	
MbVW	9-11 (10 ± 1)		
E. pore	17-54 (43 ± 10)		
Tail			45-58 (52 ± 5)
h			11-20 (16 ± 3)
Spicules		25-33 (29 ± 3)	
Gub		7.1-9.3 (8.1 ± 0.9)	

Description: *Female*: Body pear shaped with short neck and without a posterior protuberance. Head with annuli behind head cap. Stylet cone straight to slightly curved dorsally, knobs small, rounded and set off. Excretory pore 14-20 annules behind head, hemizonid just posterior to pore (Fig. 4.27). Perineal pattern rounded, dorsal arch low, lateral lines weakly visible, punctuations present in tail terminus area, sometimes striae extend laterally on one side to form a ‘wing’ (Fig 4.27 and 4.29).

Male: Head set off from the body. Head cap rounded and relatively small, labial disc not elevated, lateral lips usually not present or if present than relatively small. Head region smooth. Stylet knobs small, rounded and set off. Lateral field with four lines, a fifth incomplete linee sometimes present areolated. Hemizonid 45-58 annuli behind head, 0-4 annuli anterior to

excretory pore. Spicules slightly curved. Gubernaculum crescentic, proximal end thicker than distal end.

Juvenile 2nd: Body relatively slender. Hemizonid anterior to the excretory pore. Tail gradually tapering towards hyaline tail part, rectum often not inflated. Hyaline tail part narrow, anterior region not clearly delimited in some populations. Tail tip finely rounded, often irregularly shaped, clubbed to bifid.

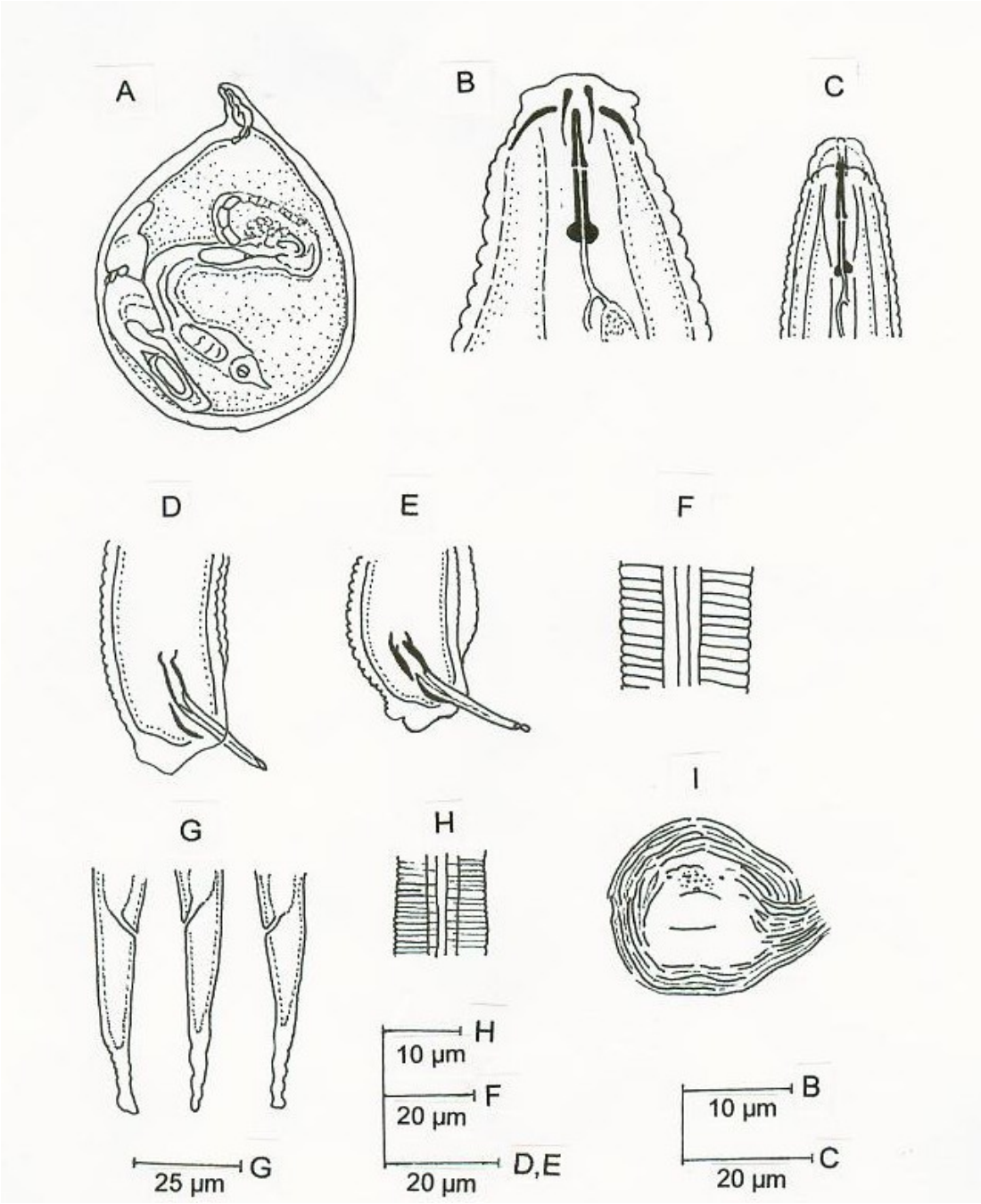

Fig. 4.27 *Meloidogyne hapla*. **A** – Female entire, **B** – Female head, **C** – Male head, **D** & **E** – Male tail variations with spicules, **F** – Mid body cuticle marking of male, **G** – Juveniles tail variations, **H** - Mid body cuticle marking of juvenile, **I** – Perineal pattern

4.4.6 *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949

(Fig. 4.28, 4.29)

= *Oxyuris incognita* Kofoid & White, 1919
= *Heterodera incognita* (Kofoid & White, 1919) Sandground, 1923
= *M. incognita incognita* (Kofoid & White, 1919) Chitwood, 1949
= *M. incognita acrita* Chitwood, 1949
= *M. incognita inornata* Lordello, 1956
= *M. acrita* (Chitwood, 1949) Esser *et al.*, 1976
= *M. elegans* da Ponte, 1977
= *M. grahami* Golden & Slana, 1978
= *M. incognita wartellei* Golden and Birchfield, 1978
= *M. incognita grahami* (Golden & Slana, 1978) Jepson, 1987

Measurements:

TABLE 4.14 Morphometrics of *Meloidogyne incognita* (measurements in µm)

N	17 ♀♀	12 ♂♂	22 J_2
L	521-748 (610 ± 94)	1148-1830 (1500 ± 214)	322-410 (360 ± 22)
W	323-510 (420 ± 80)		
a		30-51 (42 ± 7)	24-31 (28 ± 2)
b”			
c		73-197 (125 ± 40)	7-10 (8.5 ± 1.2)
c'			
Stylet	13-16 (14 ± 1)	23-32 (26 ± 0.3)	9.5-11 (10.1 ± 0.6)
SbW	3-5 (4 ± 0.7)	4.7-6.8 (5.4 ± 1.2)	
DGO	2-4 (3 ± 0.7)	1.5-2.5 (2.1 ± 0.3)	
MbL	36-62 (45 ± 8)	14-25 (19 ± 3)	10-13 (11.3 ± 0.6)
MbW	29-47 (38 ± 6)	8.6-16 (11.2 ± 4)	5.8-8.3 (7.3 ± 0.7)
MbVL	13-16 (14 ± 1)	5.8-9.1 (7.1 ± 1.3)	3.6-6.3 (5.1 ± 0.8)
MbVW	11-13 (12 ± 1)		
E. pore			
Tail			38-55 (46 ± 7)
Spicules		29-40 (35 ± 3)	
Gub		9.4-13.3 (11.1 ± 0.9)	

Description: *Females*: Body spherical with projecting neck. Head with 2 or occasionally 3 annules behind head cap. Cuticle thickening abruptly at base of relaxed stylet. Stylet knob rounded or drawn out laterally. Excretory pore at level of or posterior to stylet knobs, 11-19 annuli behind head (Fig 4.28). The perineal pattern had typical incognita type with striae closely

spaced, very wavy to zig-zag especially dorsally and laterally (Fig. 4.28 and 4.29). Dorsal arch high, rounded. Lateral field not clear, sometimes marked by breaks in striae.

Males: Head not offset, a high truncate cone shaped, clearly annulated. Head cap with stepped outline in lateral view. Stylet knobs prominent, usually with greater width than length, flat, concave or toothed anterior margins. Hemizonid usually 0-5 annuli anterior to excretory pore. Lateral field with four incisures, outer band areolated and inner band rarely cross-striated except at posterior end. Tail bluntly rounded, terminus unstriated. Spicules slightly curved, gubernaculums crescentic.

Juveniles: Head not offset, truncate cone shaped in lateral view, sub-hemispherical in dorso-ventral view. Head cap wide. Stylet knobs prominent, rounded. Hemizonid 3 annuli long anterior to excretory pore. Lateral field with four incisures, outer band cross-striated. Tail tapering to subacute terminus.

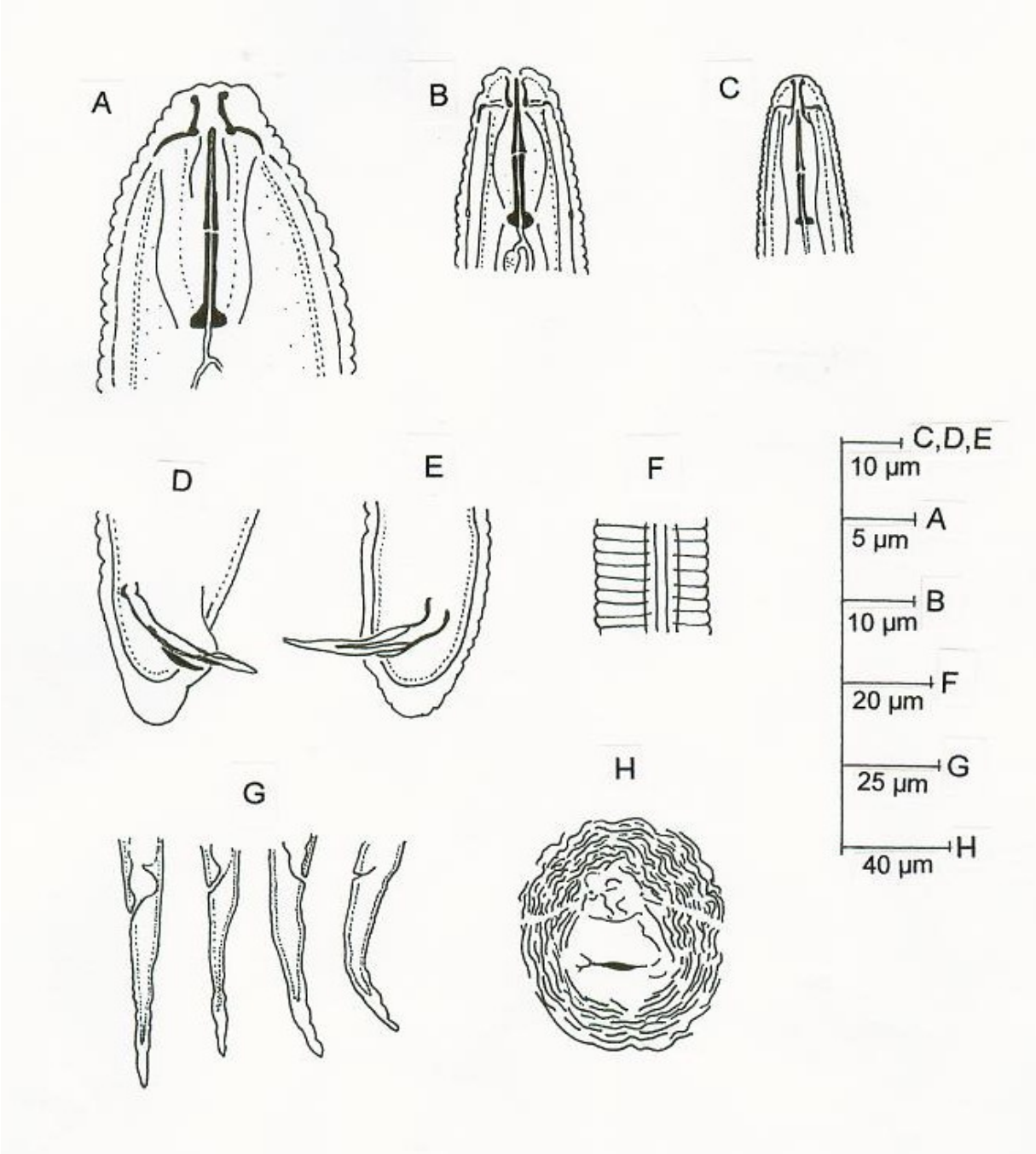

Fig. 4.28 *Meloidogyne incognita*. **A** –Female head, **B** –Male head, **C** – Juvenile head, **D** & **E** – Male tail variations with spicules, **F** – Mid body cuticle marking of male, **G** – Juveniles tail variations, **H** - Perineal pattern

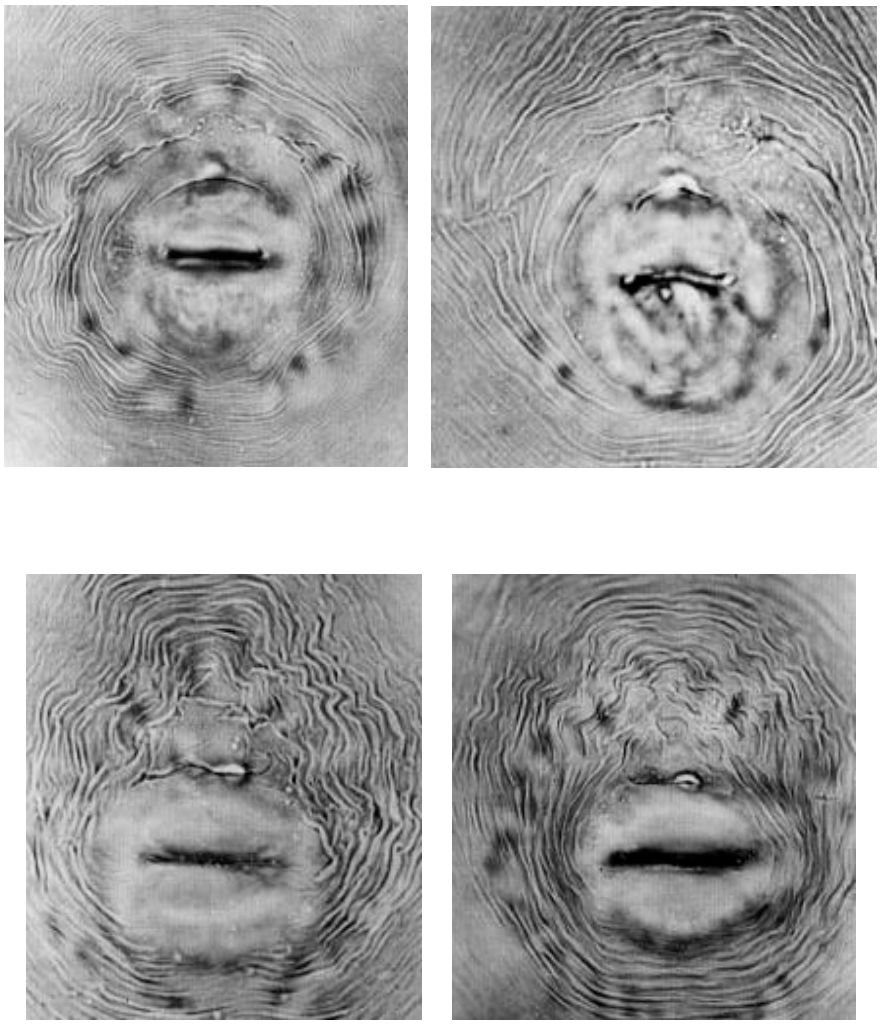

Fig. 4.29 Perineal patterns of *Meloidogyne hapla* (Upper panel) and *M. incognita* (Lower panel).

4.5 Interaction between *Pythium ultimum* and *Pratylenchus penetrans*

The fresh and dry weight of carrot under the infestation and/or infection of *Pratylenchus penetrans* alone (nematode only, N), first *Pratylenchus penetrans* and two week later *Pythium ultimum* (first nematode then fungus, NF), first *Pythium ultimum* and two week later *Pratylenchus penetrans* (first fungus then nematode, FN), *Pythium ultimum* alone (fungus only, F) and control without any pathogen (C) 94 days after seed sowing are given in Table 4.15.

In all experiments damage on carrots were significantly higher ($P < 0.001$) when inoculated with either pathogen alone or both pathogen together than the control where no pathogens were inoculated (Table 4.15). In case of single pathogen inoculum, *Pratylenchus penetrans* caused significantly more damage on carrot than *Pythium ultimum* (23% yield reduction caused by *Pratylenchus penetrans* versus 11% caused by *Pythium ultimum*).

TABLE 4.15 Fresh and dry weight of carrot under the infestation and/or infection of *Pratylenchus penetrans* only (nematode only, N), First *Pratylenchus penetrans* and two week later *Pythium ultimum* (NF), first *Pythium ultimum* and two week later *Pratylenchus penetrans* (FN), *Pythium ultimum* only (F) and control without any pathogen (C) 94 days after seed sowing.

Treatments	Carrot weight (g ± SE)	
	Fresh weight	Dry weight
C (No pathogens)	32.4 ± 3.0	4.54 ± 0.52
F (*Pythium ultimum* only)	28.8 ± 2.8	4.04 ± 0.48
N (*Pratylenchus penetrans* only)	24.8 ± 3.5	3.48 ± 0.41
FN (first *Pythium ultimum* and two week later *Pr. penetrans*)	19.7 ± 3.0	2.71 ± 0.48
NF (First *Pratylenchus penetrans* and two week later *Pythium ultimum*)	18.0 ± 3.1	2.53 ± 0.52

The damage was significantly highest (44%) when *Pratylenchus penetrans* was inoculated first and *Pythium ultimum* was inoculated two weeks after the nematode inoculum (Fig. 4.30 and Table 4.16). There was no influence of preceding pathogen when both were present. This was evident as there were no significant different on yield reduction of carrot when

both pathogen were present (Fig. 4.30). In the Fig. 4.30, there was no difference on treatment NF or FN. In the experiments where both *Pythium ultimum* and *Pratylenchus penetrans* was inoculated, no matter which one was first inoculated, the yield reduction on carrot was significantly lower than the yield reduction caused by either *Pythium ultimum* or by *Pratylenchus penetrans* alone (Fig. 4.30).

TABLE 4.16 Analysis of variance table showing *F*-value and significant different of treatments on dry weight of carrot.

	Sum of Squares	df	Mean Square	*F*-value	Sig.
Between Groups	29.20	4	7.301	33.83	0.001
Within Groups	9.71	45	0.216		
Total	38.92	49			

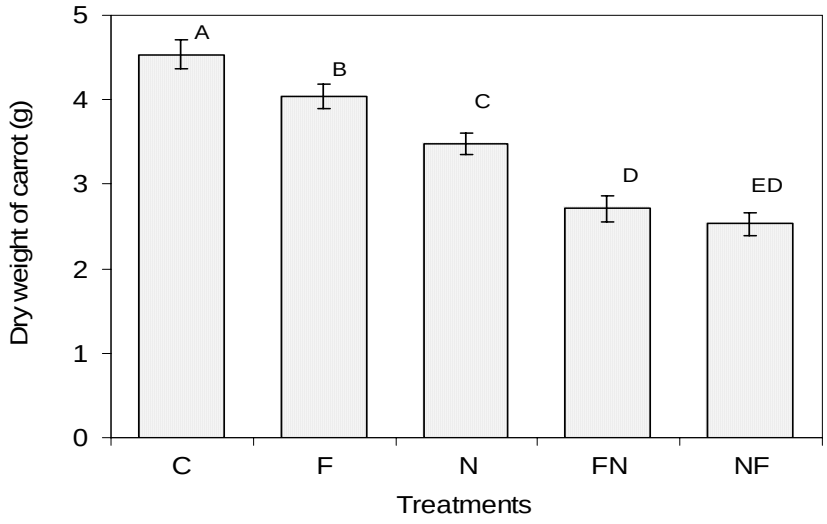

Fig. 4.30 Dry weight of carrot in gram under the infestation and/or infection of *Pratylenchus penetrans* only (N), First *Pratylenchus penetrans* and two week later *Pythium ultimum* (NF), first *Pythium ultimum* and two week later *Pratylenchus penetrans* (FN), *Pythium ultimum* only (F) and control without any pathogen (C) 94 after seed sowing. The vertical line on bar shows the standard error of means. The bars followed by common letters are not significantly different according to Duncan's multiple range tests ($P \leq 0.05$).

In percentage figure, treatment NF, FN, N and F caused 44, 40, 23 and 11% damage on carrot yield, respectively. The highest damage caused by first inoculum of *Pratylenchus penetrans* and second inoculum of *Pythium ultimum.* An interactive effect was also found that the damage caused by both nematodes and fungus together was higher than fungus or nematode alone. Fungus caused 11% yield reduction while fungus and nematode together caused 40-44% yield reduction (Fig. 4.30). The synergistic interaction was existed between *Pratylenchus penetrans* and *Pythium ultimum* as damage caused by both pathogens together was significantly higher than the summation of damage caused by either pathogen alone. *Pratylenchus penetrans* alone caused 23% yield reduction and *Pythium ultimum* alone caused 11% yield reduction, therefore the summation figure is 34% which was less than 40-44% damage caused by both pathogens together as explained above.

CHAPTER 5

Discussions

The presence of pathogenic soil fungi and plant parasitic nematodes in the same niche at the same time is very important from the point of view of their possible synergistic interaction on the disease development. It is therefore, very important to know the identification and distribution of these species for the given crops. Such information plays a key role in the decision support system for the integrated disease management programme. In this study nine species of fungi belonging to four genera and six species of nematodes belonging to four genera were recorded (See Table 4.1).

In *Pythium* genus, four species were identified based on morphological description and morphometrics. They are namely *Pythium aphanidermatum, P. ultimum, P. irregulare* and *P. sulcatum*. *P. aphanidermatum, P. ultimum* and *P. irregulare* are cosmopolitan pathogens with a wide host range. They are aggressive species of *Pythium*, causing damping off, root and stem rots, and blights of grasses and fruit (Lehman and Wolf, 1926). They are of economic concern on most annuals, cucurbits and grasses. *P. aphanidermatum* is considered one of the water moulds because it survives and grows best in wet soils. Warm temperatures favour the pathogen, making it an issue in most greenhouses. In this study they were found in warm climates only (Belhara and Jeetpur). *P. ultimum* occurs in cool to moderately warm climates and found in higher density in Basantapur and Sindhuwa, a cold climate area. *P. ultimum* is one of the commonest *Pythium* species in the soil (Stanghellini and Hancock, 1970; Lumsden et al, 1975; Plaats-Niterink, 1975). *P. ultimum* was also isolated from carrot by Robertson in 1980. *P. irregulare* and *P. sulcatum* are common soil and plant inhabiting species recorded from carrot. *P. sulcatum* was also shown to be pathogenic to carrots, causing brown discolorations, root die-back and tap-root forking (Howard and Williams, 1974; Barr and Kemp, 1976; Kalu *et al.*, 1976; Howard et al, 1978).

Some of the isolates come close to a group of species, *P. aphanidermatum* (Edson) Fitzp. 1923; *P. deliense* Meurs, 1934 and *P. indigoferae* Butler, 1907 which are characterized by the possession of inflated filamentous sporangia, often intercalary antheridia and aplerotic oospores (see Fig. 4.6 and 4.7). The isolates from the present study differ from *P. deliense* and *P.*

indigoferae by having bigger oogonia and straight oogonial stalks (vs. smaller oogonia and oogonial stalks curve towards the antheridia in *P. deliense* and *P. indigoferae*). They match with *P. aphanidermatum* which is clearly distinct from other *Pythium* species on the basis of morphology and morphometrics of the sporangia, oogonia, antheridia, the apluerotic oogonium (Fig. 4.7 A), (oospore doesn't fill the oogonium) and the intercalary (rarely terminal) attachment of the antheridia. Based on the matching of the morphological description and morphometrics of Nepalese isolates with the original description given by Edson (1915), Subramaniam (1919) and based on the key given by Plaats-Niterink (1981) and Nepalese isolate is identified as *Pythium aphanidermatum* (Edson) Fitzp., 1923.

Following the same key presented by Plaats-Niterink (1981), some isolate comes close to *P. ultimum* Trow, 1901 and can be distinguished easily from its congeneric species by the combination of morphological as well as morphometrical data such as sac-like, small, stalked, monoclinous antheridia, terminal oogonia, a thick oospore wall and globose hyphal swellings.

Based on the morphological and morphometrical data, other Nepalese isolate was identified as *P. irregulare* Buisman, 1927 which was characterized by its ornamented oogonia, irregular in shape and showed a great variation in size. Their antheridia were usually monoclinous and in water cultures often falcate. Mostly 1-2 antheridia were present per oogonium, originating at some distance from the oogonial stalk, but in rare cases sessile or hypogynous antheridia occurred. In water cultures the oogonia had a stronger tendency to form projections than on solid media. Most oospores were aplerotic but often a number of plerotic or nearly plerotic oospores were also present. Based on these characters, Nepalese isolated differs from its congeners *P. ultimum* and *P. paroecandrum*. Following the key present by Plaats-Niterink (1981), Nepalese isolate is concluded as *P. irregulare* Buisman, 1927.

Based on the morphological and morphometrical data, some of the Nepalese isolate comes close to *P. sulcatum* Pratt & Mitchell, 1973. They seem to resemble *P. scleroteichum* due to their rather similar, complicatedly furrowed antheridia, but differ having even more irregular antheridia in Nepalese isolates. Moreover, the oogonia and oospores of Nepalese isolates are considerably smaller than those of *P. scleroteichum*, and it has hyphal swellings, a lower growth rate and filamentous, non-inflated sporangia. Zoospores are produced at about 20° C in Nepalese isolates, whilst in *P. scleroteichum* no sporangia are known. Other species with filamentous sporangia do not form such complicated antheridia. Based on these characteristics and also

according to the key of Plaats-Niterink (1981), Nepalese isolate is concluded as ***P. sulcatum*** Pratt and Mitchell, 1973.

In *Fusarium*, three species were identified, namely, *Fusarium oxysporum, F. solani* and *F. moniliformae*. Overall, the distribution of *F. oxysporum* is known to be cosmopolitan from arctic tundra's (Cooke and Fournelle, 1960) to alpine pasture (Luppi-Mosca, 1960). In this study, the higher density of this species was also recorded from cooler areas (Basantapur, Sindhuwa). The species is divided into many *formae speciales* (f. sp.) that can not be distinguished using morphological criteria. However, the different special forms (f. sp.) of *F. oxysporum* often have varying degrees of distribution. *F. oxysporum* and its various formae speciales have been characterized as causing the various symptoms such as vascular wilt, yellows, corm rot, root rot, and damping-off. The most important of these is vascular wilt. Of the vascular wilt-causing Fusaria, *F. oxysporum* is the most important species (Agrios, 1988; Smith *et al.*, 1988). *F. solani* has a worldwide distribution especially in agricultural soil of the warmer zones and on numerous host plants (Domsch and Gums, 1970). In Eastern Nepal, *F. solani* was also found in high density in warmer region such as Belhara and Jeetpur. The fungus has been recorded from rhizosphere of numerous cultivated and wild plants. They cause great reduction of root system, root rot and leaf chlorosis. *F. moniliformae* are widely distributed on numerous plants in tropical and subtropical countries and common in arable soil (Domsch and Gams, 1970; Dutta and Ghosh, 1964).

Based on the colour and growth of colony on PDA and on characteristics of microconidia, macroconidia, chlamydospores, some of the Nepalese isolates resemble with the original description of *F. oxysporum* given by Schlechtendahl (1824) while other isolates with the original description of *F. moniliformae* given by Sheldon (1904) or *F. solani*. Nepalese isolates having the colour of colonies that were pigmented with a reddish purple colour and surmounted by a pinkish white aerial mycelium and having short monophialides were identified as *F. oxysporum* Schelechtendahl, 1824. Other isolates having the cream coloured colonies and containing long monophialides were identified as *F. solani* (Martius) Appel et Wollenw. The other populations that were identified as *F. moniliformae* Sheldon, 1904 come close to *F. solani*, *F. oxysporum* and *F. proliferatum* (Mats.) Nirenberg. These populations were different from *F. solani* by having medium length of conidiophores and absence of chlamydospores (*vs.* longer conidiophores and presence of chlamydospores in *F. solani*); different from *F. oxysporum*, by

having longer conidiphores, by forming microconidia in chains and absence of chlamydospores (vs. shorter conidiophores, microconidia not in chains and presence of chlamydospores in *F. oxysporum*); and from *F. proliferatum* by the absence of polyphialides in the aerial mycelium (*vs.* presence of polyphialides in *F. proliferatum*). Following the synoptic key presented by Nelson *et al.* (1983) and with the help of computer based Fuskey (Seifert, 1996); the Nepalese isolates were identified as *F. oxysporum* Schlechtendahl, 1824, *F. solani* (Martius) Appel et Wollenw and *F. moniliformae* Sheldon, 1904.

Rhizoctonia solani are widely distributed and cause various types of diseases (Domsch *et al.*, 1980). They primarily attack below ground plant parts such as the seeds, hypocotyls, roots and above ground plant parts such as pods, fruits, leaves, stems. The most common disease are root rot and damping-off are world widely distributed (MacNish and Neate, 1996; Guiterrez *et al.*, 1997). The fungus occasionally infects fruit and leaf tissue located near or on the soil surface due to the mycelium and/or sclerotia. *R. solani* is a basidiomycete fungus that does not produce any asexual spores (called conidia) and only occasionally the fungus may produce sexual spores (basidiospores). These basidiospores also serve as a source for rapid and long distance dispersal of the fungus.

The morphological description of Nepalese populations resembles with the original description presented by Khün (1858) and re-description given by Sneh *et al.* (1996). Nepalese population which was characterized by wider main runner hyphae (> 7 µm), by brown hyphal pigmentation, by more than two CNN to the tips in young hyphae, by irregular shaped, light to dark brown sclerotia that is not differentiated into rind and medulla and by the absent of clamp connections brings the isolate close to *R. solani* but not to other species of *Rhizoctonia*. Furthermore, the key given by Sneh *et al.* (1991, 1996) also concluded Nepalese isolate as *R. solani* Khün, 1858.

Sclerotinia sclerotiorum is a major soil borne plant pathogen. *S. sclerotiorum* infects nearly 400 plant species and causes economic damage to a wide range of crops (Steadman *et al.*, 1994). *S. sclerotiorum* is responsible for causing root, crown, and stem rots on various plant hosts. *S. sclerotiorum* is most prevalent in cool moist regions (Farr *et al.* 1989). The most obvious symptom is the appearance of white fluffy mycelial growth that will later produce sclerotia (Agrios, 1997). The fungus infects and produces mycelium at the base of the plant that will eventually move up the stem causing it to rot.

S. sclerotiorum was best identified by observation of the ascoma. The ascoma was apothecioid in shape and yellow-brown to tan in colour. Apothecia were not formed on a stroma, rather they arose from a sclerotium produced by mature hyphae. Apothecia produced asci that stained blue when exposed to iodine. Sclerotia formed by this fungus were large, irregular in shape, and dark-brown to black in colour. This fungus is further identified as *Sclerotinia sclerotiorum* (Lib.) de Bary on plant material when they produced a white fluffy mycelium as described by Hanlin (1998).

Rotylenchus uniformis is common migratory ectoparasites of roots and found associated with carrot ((Reed, 1976). They cause wilting of plant and reduction on plant yield by feeding plants' feeder fine roots and widely distributed.

The morphological and morphometrical characters of Nepalese population come close to the *Rotylenchus uniformis* described by Castillo *et al.* (1993). However, the population is the longest species (avg. 1.42 mm in length), which resembles with longest species of *Rotylenchus* such as *R. brevicoudata* (Hopper, 1959) Brzeski & Choi, 1998; *R. cazorlaensis* Castillo & Gomez Barcina, 1988; *R. fabalus* Baydulova, 1984; *R. laurentinus* Scognamiglio & Talamé, 1973; *R. megastylus* (Baldwin & Bell, 1981) Brzeski & choi, 1998; *R. mesorobustus* Zacanda, 1985; *R. sphaerocephalus* (Baldwin & Bell, 1981) Brzeski & choi, 1998; *R. truncocephalus* Van den Berg & Heyn, 1974. Nepalese population is different from *R. brevicoudata*, *R. laurentinus* and *R. truncocephalus* by having longest stylet and from *R. megastylus* and *R. sphaerocephalus* by having overlapped pharyngeal gland. Nepalese population is clearly different from *R. cazorlaensis*, *R. fabalus* and *R. mesorobustus* by having lip annuli irregularly divided by longitudinal striae. Following the key present by Geraert and Barooti (1996), and based on the morphological and morphometrical characters, Nepalese population is identified as *R. uniformis* (Thorne, 1949) Loof & Oostenbrink, 1958.

Helicotylenchus dihystera (Cobb, 1893) Sher, 1961 is the most widely distributed and polyphagous species in its genus. It was first described by Cobb as *Tylenchus dihystera* in 1893 from sugarcane soil, Harwood, Australia without any illustrations.

The collected populations of *Helicotylenchus* comes close to *H. dihystera*, *H. exallus* Sher, 1966 and *H. pseudorobustus* (Steiner, 1914) Golden, 1956 but differ from *H. exallus* by having an empty spermatheca and from *H. pseudorobustus* by having a shorter stylet (24µm ± 0.6 *vs.* 27.1µm ± 0.6), more posterior position of vulva (64 % ± 1.1 *vs.* 61.6 % ± 1.8) and fusion

pattern of inner lines of lateral field on tail that gives y shape (Figs. 4.24, F) compared to an u, μ or m shape in *H. pseudorobustus* (data from variability studies by Fortuner *et al.*, 1984). The description and morphometrical data of the Nepalese populations agree with the topotype of *H. dihystera* (Sher, 1966). On the basis of these characters, the Nepalese populations are identified as *H. dihystera*.

In *Pratylenchus*, two species, *Pratylenchus penetrans* and *P. neglectus* were recorded. *P. penetrans* is widespread, mainly in the temperate climates (Loof, 1991) and associated with nearly 350 host crops (Mai *et al.*, 1977). They are reported from many parts of the world such as Europe, India, Japan, Philippines, Russia and USA. It is a major pest of fruit and conifer nurseries, tobacco, apple, cherry and roses (Corbett, 1973). It is associated with carrot (Kimpinski and Thompson, 1990; Potter and Olthof, 1993). *P. neglectus* is found in temperate regions and has been reported in Europe, Canada, United States, Australia, Japan, South Africa and north-western India. They are associated with carrot (Siddiqi *et al.*, 1973).

The morphological and morphometrical characters of Nepalese populations comes close to *P. penetrans* (Cobb, 1917), Filipjev & Schuurmans Stekhoven, 1941; *P. crenatus* Loof, 1960; *P. flakkensis* Seinhorst, 1968; *P. neglectus* (Rensch, 1924), Filipjev & Schuurmans Stekhoven, 1941 and *P. psedopratensis* Seinhorst, 1968. Some of the Nepalese population is different from *P. flakkensis* by having smooth tail tip *vs.* coarsely and irregularly striated tail in *P. flakkensis*. The presence of ampula near the opening of excretory-secretory pore in our population clearly differentiates them from *P. crenatus* which is absent in *P. crenatus*. These population was also different from *P. neglectus* by having spermatheca full of sperms (*vs.* empty spermatheca in *P. neglectus*); from *P. psedopratensis* by having rounded spermatheca (*vs.* oval spermatheca in *P. psedopratensis*). The morphological and morphometrical characters of Nepalese population very much resembles with the description of *P. penetrans* given by Corbett (1973).

Other Nepalese populations was different from *P. penetrans*, *P. flakkensis* and *P. psedopratensis* by having empty spermatheca; different from *P. crenatus* by having smooth tail terminus (*vs.* crenated tail terminus in *P. crenatus*). The morphological and morphometrical characters of these populations comes close to the *P. neglectus* by having empty spermatheca and 'head cap' formed by the fusion of dorsal and ventral sub-median lips as shown by Corbett and Clark (1974) and Sher and Bell (1975). Based on the morphological intraspecific variability used in identification of *Pratylenchus* species given by Loof (1991) and following the key of

Handoo and Golden (1989), Nepalese population are identified as *P. penetrans* (Cobb, 1917), Filipjev & Schuurmans Stekhoven, 1941 and *P. neglectus* (Rensch, 1924), Filipjev & Schuurmans Stekhoven, 1941.

In *Meloidogyne*, two species namely, *Meloidogyne hapla* and *M. incognita* were identified. *M. hapla* has been referred to as the Northern Root-Knot Nematode because it commonly occurs in cooler environments. It is also found in the tropics and subtropics at higher elevations (Orton Williams, 1974). *M. hapla* has a wide host range with over 550 host species or varieties (Goodey *et al.*, 1965) which does not include graminaceous species. The galls induced by the nematode are usually smaller than those produced by *M. arenaria*, *M. incognita* and *M. javanica* and often have fine roots growing out of them. *M. incognita* found in the tropics and subtropics (Orton Williams, 1973) and has a wide host range with over 700 host species or varieties (Goodey *et al.*, 1965). It is never found where, average monthly temperatures approach freezing. The galls induced by the nematode vary in size from small to large (Byrne *et al.* 1977). Both species are found associated with carrot and cause more than 50% yield reduction (Wilson, 1957; Potter and Olthof, 1993).

The morphology and morphometrics of some of the population matches with that of *M. hapla* Chitwood, 1949 described by Orton Williams (1974), Jepson (1989) and Eisenback and Hirschmann (1991). The male head cap was rounded in the population from the present study like those of *M. hapla* which did not extend as far posteriorly as the other common species of *Meloidogyne*. The perineal pattern of our population had wing and general rounded pattern with distinct punctations near the tail which is the distinguishing character of *M. hapla*. The juvenile length ranges from 312-330 µm, and the stylet is fine with small, rounded knobs. The galls produced on carrot roots were small and fine roots were growing out of them (Christie, 1932). Based on these characters the population from the present study is identified as *M. hapla* Chitwood, 1949.

The morphology and morphometrics of other population matches with that of *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 described by Orton Williams (1973), Jepson (1989) and Eisenback and Hirschmann (1991). The male had large, rounded head cap in our population like those of *M. incognita*. The perineal pattern of our population had incognita type with striae closely spaced, very wavy to zig-zag especially dorsally and laterally and a high, squarish dorsal arch which is a distinguishing characteristics of *M. incognita*. The

juvenile length ranges from 322-410 µm, and the stylet is fine with prominent, rounded knobs. The galls produced on carrot roots were very large. Based on these characters our population is identified as *M. incognita* (Kofoid & White, 1919) Chitwood, 1949.

After the identification of nematodes and fungi, the further study was carried out on the interactive effect of these pathogens together in the disease development in carrot. Both *Pythium ultimum* and *Pratylenchus penetrans* are important pathogens that can cause yield reduction of carrot (Robertson, 1980; Mildenhall *et al.* 1971; Potter and Olthof, 1993, Talavera *et al.*, 2001). *Pythium ultimum,* if it does not kill plants outright, it leads to poor root development, stunting and reduced yields (Hendrix and Campbell, 1973). An interesting disease of muck grown carrots, characterized by brown rot and froking, proved to be caused by *P. ultimum* (Mildenhall *et al.* 1971). Root infection by *Pratylenchus penetrans* reduced carrot growth (Talavera *et al.*, 2001). In organic soil, initial densities between 100-400 *P. penetrans* /100 ml soil reduced growth of the carrot taproot considerably and induced heavy branching when densities were between 200-400 *P. penetrans*/100 ml soil (Vrain and Belair, 1981). Similarly, Potter and Olthof (1993) also calculated threshold range for *P. penetrans* in carrot is 30 to 180/100 g soil at planting, with moderate damage at about 100/100 g. Damage such as retarded growth and severe reduction on quality of carrot has also been reported frequently in the Netherlands (Koot and Kroonen-Backbier, 1999).

In this study *P. penetrans* alone caused about 23% yield reduction in carrot. This is agreed with the reports given by many workers where *P. penetrans* alone caused damage to carrot by root infection (Potter and Olthof, 1993, Talavera *et al.*, 2001) or by heavy branching of tap root (Vrain and Belair, 1981). The fungus *Pythium ultimum* alone also caused about 11% yield reduction of carrot. Mildenhall *et al.* (1971), Howard *et al.* (1978) and Liddell *et al.* (1989) also reported damage caused by *P. ultimum* in carrot.

The highest damage caused by first inoculum of *Pratylenchus penetrans* and second inoculum of *Pythium ultimum* can be attributed to the hypothesis that the stylet of nematodes puncture the root cells which became door for fungus to invade the root tissues as suggested by many authors (e. g. Oyekan and Mitchell, 1971; Manzanilla-López *et al.*, 2004). An interactive effect was also found that the damage caused by both nematodes and fungus together was higher than fungus or nematode alone. The synergistic interaction was existed between *Pratylenchus penetrans* and *Pythium ultimum* as damage caused by both pathogens together was significantly

higher than the summation of damage caused by either pathogen alone. *Pratylenchus penetrans* alone caused 23% yield reduction and *Pythium ultimum* alone caused 11% yield reduction, therefore the summation figure is 34% which was less than 40-44% damage caused by both pathogens together as explained above. Such a synergistic interaction was reported on a popular disease complex called 'Potato early dying syndrome' caused by the interaction of *Pratylenchus penetrans* and *V. dahliae* (e. g. Rowe *et al.*, 1985; MacGuidwin and Rouse, 1990; Botseas and Rowe, 1994; Saeed *et al.*, 1998). Such a synergistic interaction can be attributed to the breaking of resistance of fungi against host by nematode feeding. Similar finding has been obtained where *Fusarium oxysporum* f. *pisi* broke down resistance of peas when inoculated with *P. penetrans* (Oyekan and Mitchell, 1971).

CHAPTER 6

Conclusion

The taxonomical study and identification of the pathogenic soil fungi and the parasitic nematodes present in rhizosphere and roots of carrot from five locations of two districts of Eastern Nepal were undertaken. Nine species of fungi and six species of nematodes have been identified. The fungal species were *Pythium aphanidermatum, P. ultimum, P. irregulare, P. sulcatum, Fusarium oxysporum, F. solani, F. moniliformae, Rhizoctonia solani* and *Scleritinia sclerotiorum*. The nematode species were *Pratylenchus penetrans, P. neglectus, Meloidogyne hapla, M. incognita, Helicotylenchus dihystera* and *Rotylenchus uniformis*.

The nematode species *Pratylenchus penetrans, P. neglectus* and *Rotylenchus uniformis* are new records from Nepal.

Among the fungal species *Pythium ultimum, P. irregulare, Fusarium oxysporum, F. solani, Rhizoctonia solani, Sclerotinia sclerotiorum* were found in all samples collected from all five locations. Among the nematode species only *Meloidogyne hapla* were found in all samples. *Pratylenchus penetrans* were found in all samples except Jeetpur (Dhankuta district). The distribution pattern of *Rotylenchus uniformis* was narrow and found in few sample only.

Although the interaction between most of the fungi species and nematodes were possible, the study was limited to *Pythium ultimum* and *Pratylenchus penetrans* on disease development in carrot due to the lack of time to execute all possible studies. The study has been carried out in growth chamber. *Pythium ultimum* caused about 11% yield reduction while *Pratylenchus penetrans* caused about 23% of yield reduction in carrot. The combination of both species caused 40-44% yield reduction indicating the existence of synergistic effect on disease development in carrot.

The identification of many pathogenic soil fungi and parasitic nematodes and studies on interaction of different fungi and nematode combination such as *Fusarium-Meloidogyne*, *Fusarium-Pratylenchus* are the potential field of research which are recommended for further study. Recommendations are also made to the study of fungi-fungi as well as nematode-nematodes interaction. These studies will accumulate the information about antagonistic,

additive or synergistic reaction between the soil micro-organism which can be manipulated for integrated disease management (IDM) system within a concept of an organic farming.

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Apendixes

Processing and mounting of fungi

For the preparation of lactophenol, first of all 20 gm of crystal phenol were weighed carefully and dissolved in 20 ml of distilled water. 20 ml of lactic acid was mixed with 40 ml glycerine and finally mixed with phenol solution. The prepared lactophenol solution were stored into dropping bottle and labelled.

For the preparation of cotton blue, 0.05 gm of cotton blue was added to 100 ml of lactophenol as prepared earlier and mixed well. The cotton blue solution were poured into dropping bottle and labelled.

The fungi isolated from different general media as well as from selective media were stained with cotton blue and mounted on glass slide containing a drop of lactophenol. Finally, covered with cover slip and margin were sealed with nail polis to prevent evaporation of lactophenol. Later on these semi-permanent slides were observed under microscope and used to identify the fungi.

Processing and mounting of nematodes

Killing, fixing and processing of nematodes was done by Glycerol-ethanol solution as described by Seinhorst (1959) and modified by De Grisse (1969). 100 Tylenchs obtained either from root or from mineral soil fraction of each soil sample were picked out by means of needle and put into embryo-dish containing 5ml de-ionized water. Thereafter water was reduced into a drop and hot (ca. 70°C) solution I (formalin 4%+ anhydrous glycerine: 99+1) was poured over it. Then embryo-dish was covered by glass lid and left for overnight. The liquid in the dish was reduced and replaced in a weakpot containing 95% ethanol. The weakpot was kept inside the oven at 40°C for 24 hrs. After 24 hrs, the embryo-dish was taken out gently from the weakpot and kept inside the oven at 40°C. Afterward solution II (ethanol+anhydrous glycerine: 95+5) was

poured on every 2 hrs interval for 4-5 times. During the process the embryo-dish was partially covered to allow evaporation slowly. Finally solution III (ethanol+anhydrous glycerine: 50+50) was added to the embryo-dish and was left overnight in the oven at 40°C so that remaining ethanol was evaporated to leave the nematodes in pure glycerine. Thereafter embryo-dish was kept in a desiccator.

Identification of *Pythium* spp.

All the colonies obtained after two days culture at 25°C on selective media as described in 3.2.1.1 or 3.2.2.1 from both root and soil sample were transferred to corn meal agar (CMA) and potato-carrot agar (PCA) for the identification of *Pythium* spp. For the development of sporangia, a small piece of a culture on CMA was placed in a Petri dish in a shallow layer of water. The water used consisted of one part of sterilized fresh pond water and one part of distilled water. The grass leaves were sterilized by boiling for 10 min. Then 2 pieces of grass leaves were added to the Petri dish. Water was changed additionally which favour the production of sporangia and discharge of zoospores. After a few days, the *Pythium* colonized the grass leaf and develops zoosporangia along its margin (Plaats-Niterink, 1981). The fungi were mounted on slides in lactophenol-cotton blue. Drawings were made by using light microscope as described in 3.3.

Identification of *Fusarium* spp.

All the colony of *Fusarium* obtained in DCPA media were again cultured on both Komada's *Fusarium* selective medium (Komada, 1975) as described by Gamliel *et al.* (1996) and by Elmer *et al.* (1994) and also on Nash and Snyder's selective medium (Nash and Snyder, 1962). Dilution plate method was employed. The dishes were kept in diffuse light for incubation at 25°C as light kills *Fusarium*.

The colonies obtained from all these media were cultured on carnation leaf agar (CLA), a nutrient-poor media which facilitate developments of microscopical characters such as microconidia, macroconidia and chlamydospores. Isolates were further cultures on Komada's *Fusarium* selective medium (Komada, 1975) as well as on Nash and Snyder's selective medium

(Nash and Snyder, 1962) in order to collect information about colony morphology of particular *Fusarium* species. Cultures were kept in diffuse light for incubation at 25°C as light kills *Fusarium*.

The fungi or their structures were mounted on slides in lactophenol-cotton blue. Drawings were made by using light microscope as described in 3.3.

Identification of *Rhizoctonia* spp.

The fungi obtained from the cultures described in 3.2.1.3 and 3.2.2.3 was again cultured on selective media (Ko and Hora 1971, Hennis *et al.* 1978, Castro *et al.* 1988) for *Rhizoctonia* spp. The fungi were mounted on slides in lactophenol-cotton blue. Drawings were made by using light microscope as described in 3.3.

Identification of *Sclerotinia* spp.

The fungi obtained from the cultures described in 3.2.1.4 and 3.2.2.4 was again cultured on selective media developed by Steadman, *et al.* (1994). This medium consists of PDA amended with pentachloronitrobenzene, penicillin, streptomycin, and bromophenol blue. The medium will change from blue to yellow as colonies of *Sclerotinia* spp. develop. The fungi were mounted on slides in lactophenol-cotton blue. Drawings were made by using light microscope as described in 3.3.

Identification of root-knot nematodes

For the identification of root-knot nematodes, perennial patters were prepared. Some matured females were selected and placed in a small Petri dish. The neck of the female was cut and interior was pushed out gently. Then cut females were placed in a drop of 45% lactic acid present on the perspex sheet. About 10-20 such cuticles were placed on that perspex for 30 minutes to several hours. After that the cuticles were cut in half equatorially with a razor blade. The cuticles that harbour the perineal pattern were removed from that drop of 45% lactic acid and placed in next drop of 45% lactic acid. Then it was trimmed around the perineal pattern so that it bears square shape. About 5-10 such perineal patterns were placed in 45% lactic acid and

debris present on it were cleaned thoroughly. The cleaned perineal patterns were then transferred to a drop of glycerine on a clean microscopic slide provided with a paraffin ring. It was aligned on a strait line, anus downwards. Pressed the pattern against the glass and gently covered with cover slip on the glycerine drop. Finally it was sealed and labelled.

Potato dextrose agar (PDA) (Norris and Ribbons, 1971)

Pealed potato	200 g
Dextrose	18 g
Agar	15 g
ddH_2O	1000 ml
P^H	6-7

Corn Meal Agar (CMA) ()

Difco corn meal agar	17 g
ddH_2O	1000 ml

Boil to dissolve completely. Autoclave 15 min at 121ºC.

Potato Carrot Agar (PCA) (Plaats-Niterink, 1981)

Carrot	20 g
Potato (exclude the skin)	20 g
Bacto Agar	20 g

Dice the vegetables into small pieces. Place pieces into a beaker with aprox. 200 ml ddH2O.Boil on stir/hot plate until carrot is soft, aprox. 50-60 min.on low heat. Force slurry through a fine sieve. Add ddH2O to make 1 liter, then add agar. Mix and autoclave. While pouring plates, occasionally swirl to keep carrot/potato mix suspended.

PVPP agar (Liddle et at. 1989)

Difco corn meal agar	17 g
Pimaricine	5 mg
Vancomycine	250 mg
Penicillin	50 mg
Pentachloronitrobenzene	100 mg
ddH_2O	1000 ml

N.B.: Incubated in dark.

Dichloran Chloramphenicol Peptone Agar, DCPA (Burges *et al.*, 1988)

Peptone	15 g
K_2HPO_4	1 g
$MgSO_4.7H_2O$	0.5 g
Chloramphenicol	0.2 g
Agar	20 g
ddH_2O	1000 ml

After autoclaving add 0.002 g Dicloran (6-Dichloro-4-nitroaniline) in 10 ml ethanol.

Komada's Medium (1975)

K_2HPO_4 (or 1.3g $K_2HPO_4.3\ H_2O$)	1 g
KCl	0.5 g
$MgSO_4$ Anhydrous (or 0.5g $MgSO_4.7H_2O$)	0.25 g
L-asparagine	2 g
D-galactose	20 g
Fe-Na-EDTA	10 mg
Bacto-Agar	20 g
ddH_2O	1000 ml

Autoclave, then add to the warm media (cool to 55°C first):

Quintozene (PCNB)	1 g
Oxgall	0.5 g
$Na_2B_4O_7.10H_2O$	1 g
Streptomycin sulfate	300 mg

Adjust pH to 3.8$\pm$0.2 with a solution of 10% phosphoric acid.

Nash and Snyder's Medium (1962)

Peptone	15 g
K_2HPO_4	1 g
$MgSO_4$ Anhydrous (or 0.5g $MgSO_4.7H_2O$)	0.25 g
Bacto-Agar	20 g
ddH_2O	1000 ml

Autoclave, then add to the warm media (cool to 55*C first):

Quintozene (PCNB)	1 g
Penicillin	100 mg
Streptomycin sulfate	300 mg

N.B: Too moist plates will favor bacteria.

Carnation leaf agar (CLA) (Nelson *et al.*, 1983)

Sterialized carnation leaves	Few 1 cm² pieces
Agar	20 g
ddH_2O	1000 ml

Modified Ko and Hora medium (Hennis *et al.*, 1978; Ko & Hora, 1971; Castro *et al.*, 1988).

K_2HPO_4	1 g
$MgSO_4$.7H2O	0.5 g
KCl	0.5 g
FeSO4. 7H2O	0.01 g
$NaNO_2$	0.2 g
chloramphenicol	0.05 g
agar	20 g
ddH_2O	1000 ml

After sterilization in autoclave at 121°C/20 min, wait until the medium cool down to 50°C, then add:

galic acid	0.4 g
streptomycin	0.05 g
metalaxyl (Ridomil 2E: 25%)	0.0633 g
prochloraz (Prochloraz 38.1%)	0.005 g

PPSB-PDA (Steadman, *et al.*, 1994).

Pealed potato	200 g
Dextrose	18 g
Agar	15 g
Pentachloronitrobenzene	100 mg
Penicillin	50 mg
Streptomycine	0.05 g
Bromophenol	1.0 g
ddH_2O	1000 ml