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Identification and characterization of the pathogens associated with rhizome rot complex of ginger in Kumaon region of Uttarakhand

Avinash, K Bijendra and Shivani

Abstract
Ginger is a high value cash crop being cultivated all across the world including India. India is the highest producer and exporter of ginger. In India it is cultivated mainly in all states including Uttarakhand where it is mainly grown by small and marginal farmers on which their livelihood depends and cultivated in almost all the districts of Uttarakhand. Although it is a high value cash crop but its productivity is very low and the reason seems to be continuous use of degenerated seed rhizomes which are prone to various diseases, insect-pests and nematodes. Among these, rhizome rot caused by multitude of pathogens is a complex and one of the major limiting factors in successful cultivation of ginger. During present investigation, in order to establish the etiology of disease, infected rhizomes showing symptoms of rhizome rot were collected from the ginger growing areas of Kumaon region during crop season 2015 and brought to laboratory for isolation of pathogens. Fifteen isolates of *Fusarium* and two isolates of *Pythium* were isolated on Potato Dextrose Agar. Of the 17 isolates, isolate no. 6 of *Fusarium* sp. (Kotabagh isolate) and isolate no. 16 of *Pythium* sp. (Champawat isolate) were proved to be pathogenic under glasshouse conditions. These isolates were sent to Indian Type Culture Collection, Division of Plant Pathology, Indian Agriculture Research Institute, New Delhi for further confirmation and identification up to species level. These isolates were identified as *Fusarium acuminatum* (Ellis and Everh.,) and *Pythium aphanidermatum* (Edson) Fitzp. In addition to these, six nematodes viz; *Meloidogyne* spp., *Helicotylenchus* spp., *Pratylenchus* spp., *Tylenchorhynchus* spp., *Haplolaimus* spp. and *Criconemoides* spp. were also extracted from the soil collected from Kotabagh, Haldwani, Bindukhatta and Bazpur. During field visits maggots and white grub were also noticed in the soil near infected plants.

Keywords: Ginger, *Pythium aphanidermatum*, *Fusarium acuminatum*, insects-pest and nematodes

Introduction
Ginger (*Zingiber officinale* Rosc.) is a perennial monocotyledonous herb belonging to the *Zingiberaceae*, a family comprised of 47 genera and 1400 species (Hogarth, 2000) [11]. The crop is native to either Asia in general (Singletary, 2010) [29] or specifically to India; however, its exact origin is still unclear (Zachariah, 2008) [36]. Today ginger is cultivated worldwide throughout the subtropics and tropics where it plays an important role in agricultural economic systems in these regions (Kavitha and Thomas, 2008) [a13]. Ginger is an important commercial crop cultivated for its aromatic rhizomes which are used both as spice and medicine (Sharma *et al.,* 2010 and Nath *et al.,* 2014) [4, 17]. India is considered as "The Land of Spices" from ancient times and ginger is regarded as spice crop under the Indian Spice Act, 51. The country ranked first in terms of production, consumption and export of the ginger (Arya, 2000 and Singh, 2011) [2, 29]. Cochin ginger is produced by Kerala which is leading export variety in international market. Ginger earns a large amount of foreign exchange for the country (Tarafdar and Saha, 2007 and Nath *et al.,* 2014) [33, 17].
Besides, being a major spice, ginger has many uses in food, confectionary, gingerol, curry powders, pickles, several soft drinks, alcoholic beverages, flavouring, medicinal products, baking like in traditional ginger breads, cakes, biscuits and syrup. The ginger rhizome contains carbohydrates, proteins, fats, fiber and essential oils. It has been shown to act as an antibacterial agent (Park *et al.,* 2008 and Shaista *et al.,* 2010) [19, 24] to inhibit growth of *Escherichia coli, Proteus* spp., *Staphylococci* and *Salmonella* spp. under *in vitro* assay (Voravuthikunchai *et al.,* 2006; Stoilova *et al.,* 2007 and Bhatia and Sharma, 2012) [25]. It has also been shown to have antifungal properties, inhibiting growth of *Aspergillus* spp., *Saccharomyces* spp., *Mycoderma* spp. and *Candida* spp. (Atai *et al.,* 2009) [3]. The pungent nature of ginger is due to presence of gingerol, shogoal and zingerone while b-sesquiphellendrene and ar-curcumine are responsible for ginger flavor. Several indigenous

Varieties Maran, Kuruppampadi, Ernad, Wayanad, Himachal and Nadia and two exotic varieties China and Rio-De-Janeiro became very popular among the farmers.

Ginger is planted and traded mainly in the form of fresh rhizomes in India, China and Indonesia or in a processed form in the United States (De-Guzman and Siemonsma, 1999) [6]. India is the highest producer of ginger (0.70 million MT) followed by China (0.39 million MT), Nepal (0.22 million MT), Nigeria (0.16 million MT) and Thailand (0.15 million MT) (FAO, 2014). Its total acreage in India was 0.13 million ha with 0.66 million MT production and 4.9 t/ha productivity in 2013-2014 (National Horticulture Database, 2014) [18].

In India it is cultivated mainly in Kerala, Sikkim, Meghalaya, West Bengal, Orissa, Tamil Nadu, Arunachal Pradesh, Karnataka, Andhra Pradesh, Maharashtra, Himachal Pradesh and Uttarakhand (Kumar *et al.*, 2008; Sharma *et al.*, 2010; Dohroo *et al.*, 2012 and Nath *et al.*, 2014) [14, 25, 8].

In Uttarakhand, it has occupied 0.023 million ha and 0.23 MT production with a very low productivity (National Horticulture Database, 2014) [18]. Major ginger producing districts are Tehri Garhwal, Pauri Garhwal, Dehradun, Rudraprayag, Uttarakashi and Haridwar in Garhwal region while Nanital, Almora, Udham Singh Nagar, Bageshwar, Pithoragarh, Champawat and Chamoli in Kumaon region. More than 50 per cent of the area and production is contributed by Kumaon. It is a high value cash crop for small and marginal farmers in the state and cultivated in almost all the districts of Uttarakhand. Inspite of its cultivation in all the districts, the share of Uttarakhand is only 3.08 per cent as against 21.64 per cent of Assam.

The reason of low productivity seems to be continuous use of degenerated seed which is prone to various diseases like, rhizome rot (*Pythium* spp., *Fusarium* spp.), leaf spot (*Phyllosticta zingiberi*), nematodes such as root knot nematode (*Meloidogyne* spp.), burrowing nematode (*Radopholus similis*), reniform nematode (*Rotylenchus reniformis*), stunt nematode (*Tylenchorhynchus* spp.) and lesion nematode (*Pratylenchus* spp.) (Ramana and Eapen, 1995; Sheela *et al.*, 1995; Sagar *et al.*, 2008 and Stirling *et al.*, 2013) [22, 23, 27, 28] and insect pests such as shoot borer (*Conogethes punctiferalis*), rhizome scale (*Aspidiella hartii),* root grub (*Holotrichia* spp.) and rhizome fly (*Mimegralla coerrulelfrons*) etc. (Lodha *et al.*, 1994) [16]. Among these, rhizome rot is one of the major limiting factors in successful cultivation of ginger.

Material and methods

Survey and Collection of diseased samples

Major ginger growing areas namely; Kotabagh, Haldwani, Bindukhatta in district Nainital; Batauri, Fafar, Lohaghat in district Champawat; Gagas valley of Ranikhet in district Almora; Rawal Gaon, Phapha in district Pithoragarh, were surveyed for the incidence of rhizome rot during 2015 crop season. Symptoms of rhizome rot of ginger first appear on above ground parts at the rhizome-stem intersection or "collar" in the form of watery, brown lesions. These lesions then enlarge and coalesce, causing the stem to rot and collapse (Dohroo, 2005) [7]. On leaves, the initial symptoms caused by the basal infection appear as yellowing of the tips of older leaves in form of chlorosis. Rhizomes from diseased plants appear brown, water soaked, soft and rotten and will decay gradually (Dohroo, 2005) [7]. Plants with yellow or dead shoots and healthy plants with root, rhizome and pseudostem were collected. Soil adhering to rhizomes was also collected for extraction of nematodes from Devi Rampur in Kotabagh; Kunwarpur in Haldwani; Shantipuri in Bindukhatta and Beria Daulat in Bazpur. These samples were brought to the laboratory for isolation of associated pathogens and microscopic examination.

Sterilization of glass-wares

All the glass wares; namely Petri plates, conical flasks and culture tubes used for *in vitro* studies were sterilized in hot air oven at 160^0C for 120 minutes after being thoroughly cleaned with potassium dichromate solution and air dried.

Preparation of basal media (Potato Dextrose Agar Media)

Potato Dextrose Agar (PDA) media was used to study the growth and sporulation of the fungus. Two hundred grams of peeled potato were cut into fine pieces and boiled in 500 ml of distilled water and filtered through muslin cloth. Twenty gram of agar was dissolved in 500 ml boiling water. Potato extract was added in boiling mixture and mixed thoroughly by stirring with glass rod. Twenty gram of dextrose was added in boiling mixture and final volume of the medium was adjusted to make 1 litre. The medium was then transferred to 500 ml capacity flasks and were plugged with non-absorbent cotton plugs. The p^H of the medium was adjusted to 7.0 ± 0.2. Finally, the medium was sterilized at 15 lbs p.s.i (121.6^0C) for 15 minutes.

Slant preparation

Approximately 5 ml melted PDA per culture tube was transferred, while pouring care was taken that the medium should not touch the inner wall of culture tube. Non-absorbent cotton was used for plugging the culture tubes. The culture tubes were then sterilized at 15 lbs p.s.i (121^0C) for 15 minutes in autoclave. After sterilization culture tubes were allowed to solidify in slanted position and then stored in refrigerator for further experiments.

Isolation, maintenance of pure culture and Identification of rhizome rot pathogens

Isolation of pathogens was done from infected rhizomes or pseudostems of ginger. Rhizomes or pseudo-stems showing disease symptoms were washed thoroughly in running tab water and rhizomes or pseudostems cut into small pieces. The pieces were surface sterilized with 1% sodium hypochlorite solution for 60 seconds followed by three times washing in sterile distilled water. After drying on blotter paper samples were transferred onto the PDA plates. Culture plates were incubated at 25-27 ^{0}C for one week. Isolated colony of the pathogen was further purified by sub-culturing. Pure culture of the fungus was maintained in slants for further use.

Since, several plant pathogenic nematodes have been reported in ginger which may aggravate the rhizome rot complex. Hence, soil adhering to rhizomes was also collected for extraction of nematodes from Devi Rampur in Kotabagh; Kunwarpur in Haldwani; Shantipuri in Bindukhatta and Beria Daulat in Bazpur. Nematodes were isolated from soil collected from infected plants by "Cobb's decanting and sieving technique" (Cobb, 1918) [5]. Two hundred gram of soil sample was collected in a plastic pan A and 1litre water was added to the pan. Homogenized soil suspension was prepared by breaking the soil clods or clumps while stirring the soil suspension for 15-20 seconds with the help of glass/plastic rod. The soil suspension of plastic pan A was passed through 20 mess sieve and collected in pan B. Again the soil suspension collected in plastic pan B was passed through 100 mess sieve and collected in pan A. Further, the

soil suspension collected in pan A was passed through 325 mess sieve and collected in pan B while continuous washing with water. The residue left in 325 mess sieve was collected in a beaker and marked as 325. Double layered facial tissue paper including muslin cloth piece moulded on circular plastic tube was prepared. The residue of the 325 mesh sieve was passed over the tissue paper gently. The plastic tube assembly was kept in a bowl and more water was added so that lower end of tube touches the water level. After 18 hours, the plastic tube assembly was removed and water from bowl was collected in a beaker and examined under stereo binocular microscope for identification of nematodes**.**

The identities of the pathogens were confirmed based on spore morphology and colony characteristics of the fungus under microscope with the help of standard monograph or literature. The identification of *Fusarium acuminatum* was done on the basis of conidial characters by reffering "The *Fusarium* Laboratory Manual" (Leslie and Summerelle, 2006). *Pythium aphanidermatum* was identified by reffering "Introductory Mycology" (Alexopoulos and Mims, 1996) [1]. However, plant parasitic nematodes were identified by reffering "Principles of Nematology" (Thorne, 1961) [34].

Pathogenecity Test

Since several isolates of *Fusarium* and *Pythium* were isolated from infected rhizomes or pseudostems collected from different places, the pathogenicity tests were set up separately for each isolates under glass house condition. Uniformly three healthy rhizomes per pot were sown on 5th November, 2015. The pots were pre-filled with soil sterilized with 3 per cent formaldehyde. Plants were inoculated 60 days after rhizome had been planted in each pot. For pathogenecity tests of *Fusarium* isolates about 100 ml of conidial suspension ($4.5x10^5$ spores ml^{-1}) was poured in each pot.

While for *Pythium* isolates, inoculum of each isolate was prepared separately by placing 50g sorghum seeds in 250 ml conical flasks, soaking the seeds overnight in water, pouring off the excess water, autoclaving twice on successive days and then inoculating the seeds from culture on agar. The inoculum was used after the mycelium had fully colonized the substrate (usually 7-10 days at 25^0C). Plants were inoculated 60 days after planting with 1flask/Pot. After inoculum was added, these pots were regularly watered to maintain the soil moisture. The plants were regularly monitored for the development of rhizome rot symptoms as reported in literature (Haware and Joshi, 1974; Pegg and Sirling, 1994 and Dohroo, 2005) [14, 25, 8].

At the end of each experiment, small pieces of tissue from rotting rhizomes/pseudostems were transferred onto PDA to check the presence of *Fusarium* and *Pythium* as described earlier.

For confirmation and further identification up to species level these isolates were sent to Indian Type Culture Collection, Division of Plant Pathology, Indian Agriculture Research Institute, New Delhi.

Result and discussion

In Uttarakhand, ginger is cultivated both in Kumaon and Garhwal regions. In this state, more than half of the area and production is mainly contributed by Kumaon region. The major ginger growing districts in Kumaon region are Champawat, Nainital, Almora and Pithoragarh. Therefore, major ginger growing areas in these districts were surveyed for incidence of rhizome rot during 2015 crop season. During field visits, development of symptoms was recorded, studied and documented (Figure A).

The disease generally appeared in small patches (Fig. B). Initially the disease manifested as yellowing of the tips of older leaves, gradually spreading down the leaf blade along the margin to the sheath. Occasionally, plants with yellow shoots or shoots that had fallen over or died have also been noticed. When such infected plants were examined closely especially the pseudostem, the collar region showed water soaked to pale translucent brown colour lesion. When such plants are pulled from the soil they were easily uprooted and most of the time rhizomes remained in the soil. The rhizomes from such plants were digged out, washed and examined for symptoms. The symptoms varied from water soaked, brown to black lesions to complete rotting of the rhizomes. There was partial to complete rotting of the rhizomes (Fig. C). The rotted portion had wet and pulpy appearance. The completely rotted rhizomes were emitting the foul smell (Fig. C). In some cases, white to cream coloured maggots in rotted rhizomes (Fig. Y) and white grubs in soil near the roots (Fig. Z) have also been noticed. Plants with yellow or dead shoots, healthy plants with root, rhizomes and pseudostem were collected. Soil adhering to rhizomes was also collected for extraction of nematodes from Devi Rampur in Kotabagh; Kunwarpur in Haldwani; Shantipuri in Bindukhatta and Beria Daulat in Bazpur. These samples were brought to the laboratory for isolation of associated pathogens and microscopic examination. Isolations were made from samples collected from different locations and they were designated as seventeen different isolates. These were sub cultured, purified and maintained on potato dextrose agar medium. The isolates were further examined for their cultural and morphological characteristics.

Of the 17 isolates, two isolates had characteristics typical of *Pythium* (hyaline, coenocytic (non-septate), cottony aerial mycelium (Fig. D and E), with yellowish swollen sporangia (Fig. E and F) borne terminally on hyphae and vesicles (Fig. G) and 15 isolates had cultural and microscopic characteristics considered typical of *Fusarium* (hyaline, septate, cottony white mycelium (Fig. H and I) with septate, sickle-shaped macro-conidia (Fig. J), rounded micro-conidia (Fig. K) and chain of terminal and intercalary chlamydospores (Fig. L and M).

To determine whether these isolates are pathogenic or not? Pathogenicity tests were set up separately for each isolate under glass house conditions. Plants were inoculated 60 days after the rhizomes had been planted (Fig. N). Symptoms of the disease were noticed only in the plants inoculated with isolate 6 of *Fusarium* (isolated from samples collected from Kotabagh, Nainital) and isolate 16 of *Pythium* (isolated from samples collected from Champa wat). Plants inoculated with isolate 6 (*Fusarium* sp.) showed signs of yellowing at leaf margins after 5th day of inoculation however, in plants inoculated with *Pythium* isolates yellowing of the plants was not observed until 10th day. Later on, of the 15 replicate plants 6 and 8 plants were died on 35th day of inoculation due to *Fusarium* and *Pythium*, respectively (Fig. O). When plants were pulled out from pots and examined the basal portion of the pseudostem and parts of the rhizomes attached to pseudostem showed water soaked, dark brown to black lesions with partial rotting of rhizomes (Fig. P). The pathogens were re-isolated from infected rhizomes/ pseudostems of plants inoculated with *Fusarium* and *Pythium*, separately. Similar, cultural and morphological characteristics have been observed as recorded earlier.

For confirmation and further identification up to species level, the pathogenic isolates were sent to Indian Type Culture Collection, Division of Plant Pathology, IARI, New Delhi. These isolates were identified as *Fusarium acuminatum* (Ellis and Everh.) and *Pythium aphanidermatum* (Edson) Fitzp.

In addition, to *F. acuminatum* and *P. aphanidermatum*, six different nematodes viz.; *Pratylenchus* spp., *Meloidogyne* spp., *Haplolaimus* spp., *Helicotylenchus* spp., *Criconemoides* spp. and *Tylenchorhynchus* spp. have also been extracted from the soil adhering to the infected plants from Devi Rampur in Kotabagh; Kunwarpur in Haldwani; Shantipuri in Bindukhatta and Beria Daulat in Bazpur (Fig. R to X). Similar finding has already reported by Ramana and Eapen (1995) [22] and Sheela *et al.,* (1995) [27].

Present study indicated that the infected plants were mostly located in the low lying/wettest portion of the field and also the incidence of disease increased in July and August (rainy months). Dohroo (2005) [7] and Stirling (2009) [7] have noted that wet conditions and high soil water were the most important factors influencing the development of rhizome rot in India, Fiji and Australia. Perhaps, the higher disease incidence in July and August may be attributed to heavy rains leading to improper drainage which favour rhizome rot disease (Sagar *et al.,* 2008) [23]. Under high soil moisture conditions, zoospores disperse in soil water and spread the disease to adjacent clumps (Dohroo, 2005) [7]. Similar symptoms of rhizome rot have also been reported by several workers (Pegg and Sirling, 1994; Haware and Joshi, 1974; Dohroo *et al.,* 2012) [20, 10, 7].

Pathogenic experiments conducted during present investigations confirmed the association of *Fusarium* and *Pythium* isolates with rhizome rot samples collected from Kotabagh and Champawat, respectively. Identities of these isolates were confirmed as *Fusarium acuminatum* and *Pythium aphanidermatum* by reffering standard literature (Leslie and Summerelle, 2006 and Alexopoulos and Mims, 1996) [15, 1] and further confirmed by Indian Type Culture, Collection, Division of Plant Pathology, IARI, New Delhi. *F. acuminatum* has earlier been isolated from the ginger rhizome by Ingle *et al.* (2008) [12] but they have not proved the pathogenecity. Thus whether it was pathogenic or saprophytic not clearly reported in literature. It seems that this is the first report of *F. acuminatum* causing rhizome rot of ginger in India. However, *P. aphanidermatum* has been reported by several researchers (Rajan *et al.,* 2002; Sagar *et al.,* 2008; Dohroo *et al.,* 2012 and Nath *et al.,* 2014) [21, 23, 7, 8] from various ginger growing areas in India.

All these pathogens are known to form complexes with soil borne nematodes- *Meloidogyne* spp., *Radopholus similis, Helicotylinchus multicinctus, Rotylenchus reniformis, Pratylenchus* spp., *Tylenchorhynchus* spp., *Haplolaimus indicus, Criconemoides* spp. and *Xiphinema* spp. (Raman and Eapan, 1995; Sheela *et al.,* 1995; Sagar *et al.,* 2008 and Stirling *et al.,* 2013) [22, 27, 28, 30] and insect-pests (*Conogethus punctiferalis, Aspidiella hartii* and *Holotrichia* spp.). In present study we have also extracted 6 nematodes which were identified as *Pratylenchus* spp., *Meloidogyne* spp., *Haplolaimus* spp., *Helicotylenchus* spp., *Criconemoides* spp. and *Tylenchorhynchus* spp. by reffering standard literature (Thorne, 1961) [34] and with help of a Nematologist in the department. Besides, during field visits we noticed maggots in rotted rhizomes and white grubs in the soil adjacent to infected plants (Fig. Y and Z). They predispose the crop to secondary pathogens (Sharma, 1994) [26].

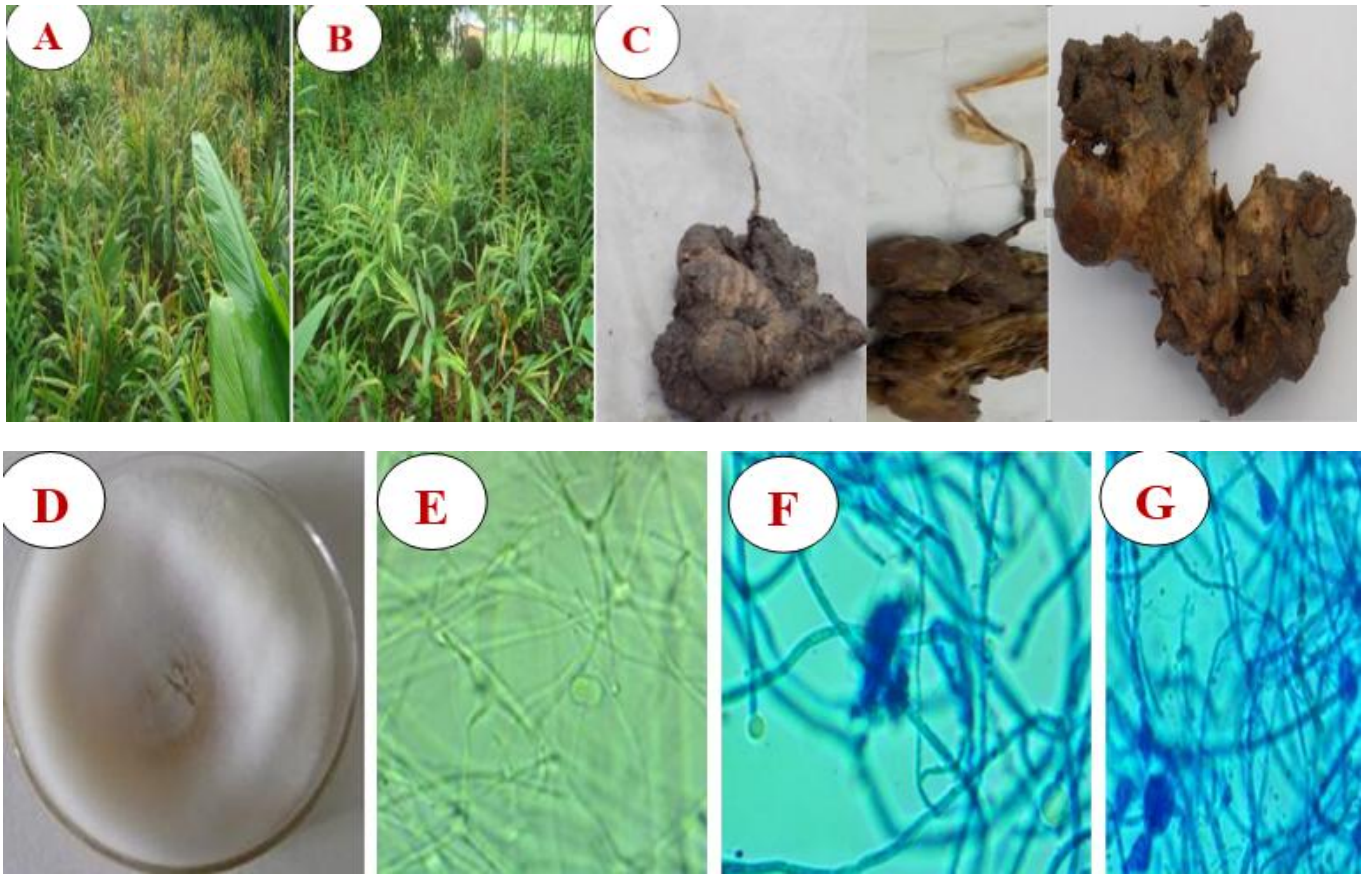

Fig: A Rhizome rot infected ginger field, B. Patchy appearance of rhizome rot of ginger, C. Rotted rhizome and pseudostem, D. Colony character of *Pythium aphanidermatum*, E and F. Coenocytic mycelium and terminal swollen sporangia, G. Vesicles (At 100X).

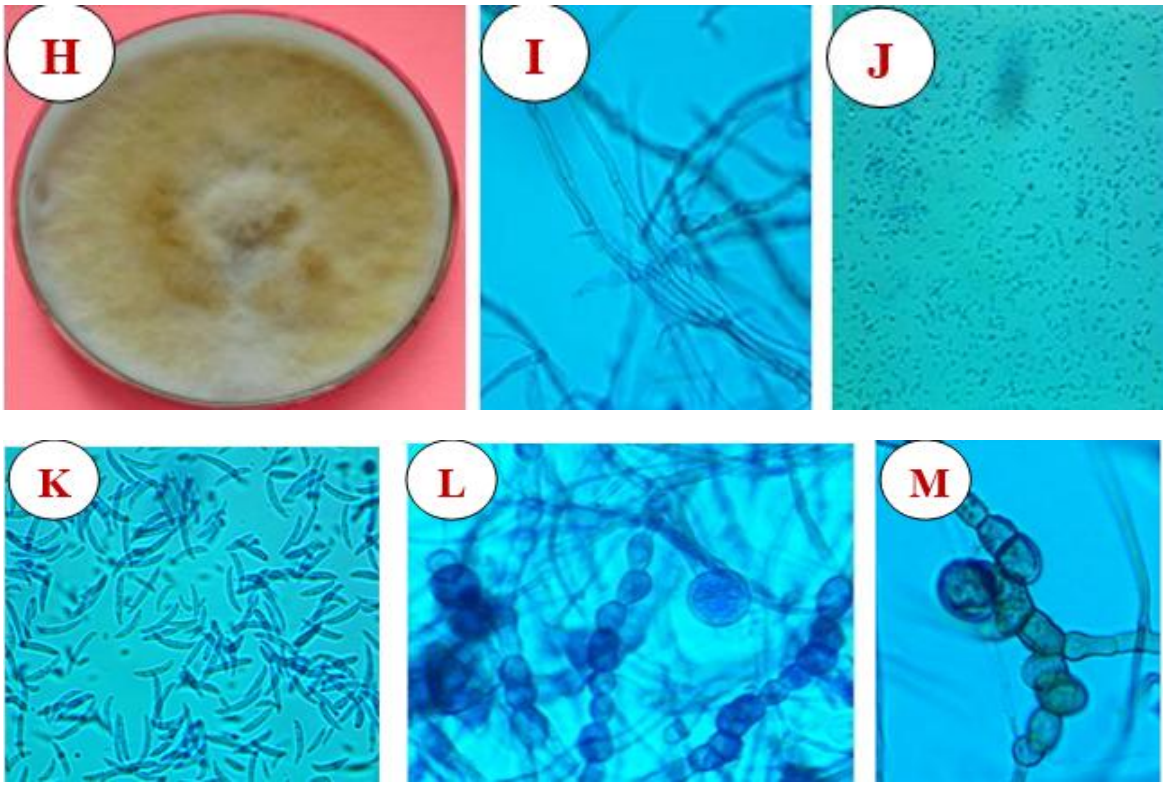

Fig: H. Colony character of *Fusarium acuminatum*, I. Septate mycelium J. Rounded micro-conidia of *F. acuminatum*, K. Sickle shaped, septate macro-conidia of *F. acuminatum*, L. to M. Chlamydospores in chains (At 100X).

Nematodes and insect-pests as associated pathogen with rhizome rot complex of ginger and Maggots and White grub

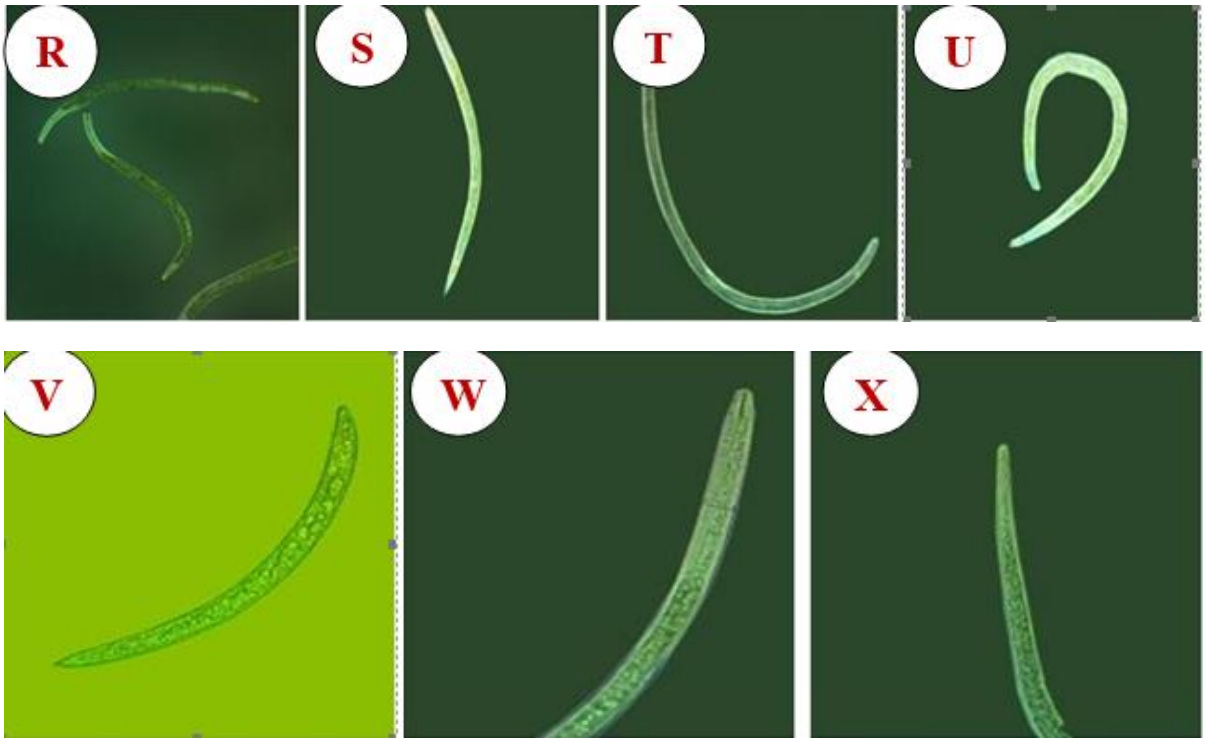

Fig N. Inoculation of rhizome, O. Initial symptoms and advance stage, P. Infected rhizome and pseudostem Q. Control, R. *Pratylenchus* spp., *Meloidogyne* spp., T. *Hoplolaimus* spp., U. *Helicotylenchus* spp., V. *Criconemoides* spp., W. *Tylenchorhynchus* spp., head region, X. *Tylenchorhynchus* spp., tail region, Y. Maggots in infected rhizomes, Z. Root grubs larvae.

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