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The Rhizome Rot and Wilt complex of ginger (*Zingiber officinale)*have a very complex etiology. In the present study attempt have been made to identify and characterize the different pathogens associated with the disease complex of ginger in Darjeeling Himalayas. *Fusarium solani* and *Fusarium moniliforme* were isolated from the infected rhizome and soil from villages of Kalimpong sub-division. Besides, *Pythium* spesies, *Ralstonia solanacearum* and two species of nematodes *viz. Pratylenchus* sp. and *Meloidogyne* sp. could also be isolated from the infected rhizome and soil. Among the two types of *Fusarium* sp., *Fusarium moniliforme* was dominating in both the soil and rhizome of 14 different villages of Kalimpong sub-division. *Ralstonia solanacearum* isolated from ginger from hill agro-climatic region of West Bengal belongs to biovar III group.

Key words: *Zingiber officinale, Ralstonia solanacearum*, *Fusarium*, wilt complex

INTRODUCTION

Ginger (*Zingiber officinale* Rosc.) is an important commercial crop of tropical and subtropical countries belonging to the family Zingiberaceae. It is grown for its aromatic rhizomes which are used both as spice and medicine. It is cultivated in almost all the tropical and subtropical parts of India, especially in Sikkim, Kerala, Karnataka, Tamil Nadu, West Bengal, Bihar, Himachal Pradesh, Uttar Pradesh and Maharashtra. Ginger is cultivated in Darjeeling hills since time immemorial. It is grown as one of the important cash crop of the area up to 1500 metres altitude. During these days the acreage and productivity of ginger in the area has declined tremendously due to the occurrence of disease having complex etiology involving fungi, bacteria and nematodes. Presences of different pathogens have been reported by several workers. Association of fungal pathogens like *Fusarium oxysporum, Pythium pleroticum, Pythium, aphanidermatum, Fusarium equiseti,* and *Fusarium solani,* bacterial pathogens like *Erwinia chrysanthemi, Ralstonia solanacearum*, *Enterobacter cloacae* and nematodes like *Pratylenchus coffeae* (Thapa *et al.,* 2008), *Meloidogyne incognita* have been reported. This paper reports association of different pathogens with the Rhizome rot and Wilt disease complex of ginger in Darjeeling Himalayas.

MATERIALS AND METHODS

Field survey and collection of samples

An extensive survey of Rhizome Rot and Wilt complex diseases was undertaken in 96 plots from 14 different villages and 103 plots from 12 different villages of Kalimpong sub-division under Darjeeling

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District of West Bengal during May-October in the years 2014 and 2015 respectively. Infected rhizomes, plant parts and rhizosphere soil from the surveyed locations were collected for isolation and characterization of the pathogen following standard methods.

Isolation of pathogenic fungi from soil

The pathogenic fungi were isolated from ginger growing soils of different location following standard soil sampling method. Ten gram (10g) of soil from the collected samples was crushed with sterilized mortar pestle and shaken with 90 ml of sterilized distilled water for 10-20 minutes to obtain a soil suspension. The soil suspensions thus obtained were used for isolation of pathogenic fungi by dilution plating method on Potato Dextrose Agar (PDA), PDA with Bavistin and *Pythium* specific medium. The plates were then incubated in incubator at 27±2^{0}C. The different colonies were picked up and purified separately hyphal tip culture technique to study colony characteristics, pigmentation, growth type, growth rate etc. the basis of colony morphologies and characters. The slides were prepared from the purified cultures and examined under microscope. Size of the various fungal structures were measured microscopically with the help of ocular and stage micrometer.

Isolation of pathogenic fungi from rhizome

The infected rhizomes were cut into pieces of about 20-30g by sterilized blade and surface sterilized with mercuric chloride solution (0.1%). The cut pieces were then washed with sterilized water and excess water was soaked by sterilized blotting paper. Such surface sterilized pieces were then placed on sterilized medium same as used in case of isolation of pathogenic fungi from soil. Morphologically different fungal growths were picked up and purified following hyphal tip culture techniques. The purified cultures of fungi isolated from soil as well as from rhizomes were microscopically examined for generic level identification. Stock of purified fungi were maintained on PDA slants and preserved in the refrigerator at 4^{0}C. Apart from weekly transfer for experimental work, cultures were tested at regular interval to evaluate pathogenicity.

Isolation of pathogenic bacteria from soils

Associated bacteria was Isolated from ginger growing soils of Kalimpong sub-division. Soil from infected ginger fields were collected by following standard soil sampling method. From the collected samples, ten grams (10 g) of soil was crushed in a sterilized mortar pestle and 90 ml of sterilized distilled water was added to it and shaken for 10-20 minutes to obtain a soil suspension. The soil suspension so obtained was used for isolation by dilution plating technique on Tetrazolium chloride (TZC) medium. These plates were incubated for 48 hr at 28±1^{0}C. After incubation, morphologically fluidal colonies with pink centre were picked up and purified (characteristic colonies of *Ralstonia solanacearum).*

Isolation of pathogenic bacteria from infected plant part

Infected pseudostems and rhizomes were cut into pieces and placed aseptically in sterilized water in test tubes. Then the bacterial suspension was streaked in quadric fashion on the Petriplates containing TZC medium. The plates were incubated at 28±1^{0}C for 24 hr and the characteristic fluidal colonies with pink centre were picked up and purified. Cultural characters of associated bacterium were studied by single stroke inoculation on *Ralstonia solanacearum* specific medium slants. For pigment production, a 24 hr growth of test bacterium was streaked on TZC slants and yeast glucose chalk agar slant and incubated at 28±1^{0}C. The observations were taken after 72 hr of incubation.

Enzymatic activity

To detect the production of catalase enzyme, a loopful of 36 hr slant growth of the test bacterium was smeared on a slide and it was covered with a few drops of 20 volume hydrogen peroxide. The reaction is positive if gas bubbles are produced. At times it is necessary to examine for gas bubbles under a microscope, if catalase activity is mild. For oxidase test, homogeneous suspension is made by mixing thoroughly a loopful of 24 hr growth of test bacterium culture by continuous stirring in 2-3 ml sterile distilled water. Then few oxidase discs were taken in a clean sterile Petri plate and one or two drop of this inoculum suspension was added to disc. In positive reaction the disc changes its colour to blue within 60 seconds.

Gram Reaction

To test for gram negativity, a loopful inoculum of 24hrs growth of test bacterium was taken on a clean slide and a drop of 3% KOH solution was mixed with the smear of the bacterium. Gram-negative bacteria appear as sticky where as gram-positive bacteria showed no stickiness. Characteristics of isolated bacterium were also studied using biochemical test kits**.**

Characterization using Kit

KB002 (Hi-Media) is a standard colorimetric identification system. In the present study it was used for characterization of the associated bacterium. A loopful of bacterial culture was taken and a homogeneous suspension is made in 2-3 ml sterile distilled water. The density of the suspension adjusted to 0.1 OD at 620 nm. This suspension was used for inoculation of the kits. Each well was inoculated with 50 ml of above inoculum suspension by surface inoculation method. Inoculated test kits were incubated at 35-37^{0}C for 18-24 hr. The tests are based on the principle of pH changes and substrate utilization. On incubation organism undergoes metabolic changes, which are indicated by a colour changes in the media that can be either interpreted visually or after addition of the reagent.

Isolation of pathogenic nematodes

For isolation of pathogenic nematodes soil sample of 250 g was taken in a plastic container and thoroughly agitated with equal amount of water and allowed to settle. Heavier soil materials precipitates and the lighter particles along with the nematodes formed a suspension. The suspension was then decanted off and passed through a series of sieves having a range of mesh from 22 to 500. Suspension containing nematodes was first poured through the sieves of coarse mesh (22) screen to remove coarse organic matter, stone particles and large soil organisms including the nematodes coiled around the root bits. The filtrate collected in another bowl was then passed through a screen of 60 mesh sieve to separate particles measuring 1 to 1.5 mm or more. The filtrate and residues were collected in different containers. The filtrate was passed through a series of sieves (250, 325, 400 and 500 mesh screens).The final collection of the filtrate was made after passing through the sieve of 500 mesh which usually separate materials measuring 0.2 to 0.5 mm. The residues deposited on the sieves of 250, 325 and 400 mesh screen were collected. This process was continued for several times and final collections were poured through moist tissue papers placed on flat aluminum screen in Petriplates. The tissue papers were kept moistened by maintaining a shallow layer of distilled water below the aluminum screen in the Petri plates touching the filter papers. Water suspensions were then examined under a stereoscopic microscope. The residue collected from the 60 mesh screen was again agitated with distilled water and passed through a series of sieves as mentioned earlier for collection of remaining nematodes through moist tissue paper.

Isolation of pathogenic nematodes from roots and rhizomes

Infected roots were washed thoroughly in running tap water, cut into smaller bits, blotted dry, mixed thoroughly. Root bits of 10 g were taken and macerated for 45 seconds in an electric blender. Macerated root were poured into the soft tissue paper supported by wire net in a Petri plate containing distilled water, lower surface of the wire mesh just touching the water. Similar process was followed for extraction of nematodes from rhizome.

RESULTS AND DISCUSSION

Associated fungal pathogens

Two different types of the *Fusarium* from the infected rhizome and soil were obtained. The morphological studies and growth pattern of two different types of *Fusarium* were presented in the Table 1. These isolates were designated as F1 and F2 type Light yellow type of pigmentation was observed in F1 type, where size of macroconidia and microconidia varied from 19.64 - 21.43 µm x 4.46 – 6.25 µm and 8.04 - 12.5 µm x 2.69 - 4.64 µm respectively. The size of the chlamydospore was 8.04 - 11.16 µm and width of hyphae was 2.69 – 3.57 µm. In case of F2 type of *Fusarium,* initially violet type of pigmentation was found and then it turned into black colour. The size of macroconidia and microconidia varied from 7.14 - 11.61 µm x 1.79 - 3.57 µm and 4.46 – 6.25 µm x 1.79 - 2.68 µm respectively. The size of the chlamydospore is 4.46 - 11.16 µm and width of hyphae was 2.68 - 3.57 µm. Both F1 and F2 types of cultures were

Table 1 : Morphological characterization of isolated *Fusarium* spp

Fungus	Growth rate/day (mm)	Growth type	Pigmentation	Size of the macroconidia		Size of the micro-conidia		Size of the Chlamydospore (µm)	Width of the hyphae (µm)
				Length (µm)	Width (µm)	Length (µm)	Width (µm)		
Fusarium solani	13.25	Yellows white with copious mycelium	Light yellow	19.64 – 21.43	4.46 – 6.25	8.04 – 12.5	2.69 – 4.64	8.04 – 11.16	2.69 – 3.57
Fusarium moniliforme	15.79	Fluffy with sparse mycelium	Initially violet then blackish	7.14 – 11.61	1.79 – 3.57	4.46 – 6.25	1.79 – 2.68	4.46 – 11.16	2.68 – 3.57

Table 2 : Morphological characteristics of isolated *Pythium* species

Fungus	Colony characteristics	Hyphae	Sporangia	Growth rate (mm / day)
Pythium sp.	White with raised colony with compact aerial mycelium	Much branched hyphae, hyaline, coenocytic	Obserbed on few branched hyphae, lobulate bud like outgrowth	8.15

Table 3: Biochemical characterization of isolated *Ralstonia* pathogen

Table 3a: Biochemical Reaction

Isolates	Biochemical Reactions									
	Gram Reaction (3% KOH)	Catalaze test	Oxidase test (Seconds)	Citrate utilization	Lysine Decarboxylation	Urease	Phenylalanine Deamination	Nitrate reduction	H_2S production	Ornithine Decarboxylation
Kalimpong I	- ve	+ ve	+ ve	+ ve	- ve	+ ve	- ve	+ ve	- ve	- ve
Kalimpong III	- ve	+ ve	+ ve	+ ve	+ ve	+ ve	- ve	+ ve	- ve	- ve

Table 3b: Carbohydrate Utilization Test

Isolates	Carbohydrate Utilization Test (Fermentation)					
	Maltose	Lactose	Mannitol	Cellobiose	Sorbitol	Dulcitol
Kalimpong I	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
Kalimpong III	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve

The bacterial pathogen

The bacterial pathogens were isolated on Tetrazolium Chloride (TZC) medium and purified cultures were used for pathogen identification and establishment of Kochs' postulate. The isolated bacterial pathogen were found to be gram negative, short rod and on TZC medium the isolates produced fluidal, slightly raised colony with pink center surrounded by the white fluidal mass after 48 hrs of incubation. The isolates were oxidase and catalase positive. All the isolates were arginine dihydrolase negetive and no growth was observed at 4^0C and 40^0C temperature. Besides, all the isolates were able to reduce nitrate (Table 3a). The isolates were characterized for biovar determination. and Kalimpong ginger isolate of *Ralstonia solanacearum* were found to be Biovar III as they were able to utilize cellobiose, lactose, maltose and

Table 4: Morphological characteristic of isolated nematodes species

Nematodes species isolated	Total body length (mm)		a		b		c		v		Spear length (µ)	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Pratylenchus sp.	0.65	0.65	36.0	31.0	6.0	6.0	23.0	22.0	50.0	78.0	17.0	17.0
Meloidogyne sp.	-	0.7	-	-	-	-	-	-	-	-	-	36
Meloidogyne sp. (Larvae)	0.5		28.0		7.0		9.0		-		29.0	

a = Body length/Width **b** = Body length/Oesophagus **c** = Body length/Tail **v** = Vulvular distance/Total length of the body
♂ - Male ♀ - Female

sent to Agharkar Research Institude, Pune, Maharashtra, for species level identification. F1 was identified as *Fusarium solani* and F2 as *Fusarium moniliforme*. Among the two types of *Fusarium* sp., *Fusarium moniliforme* was dominating in both the soil and rhizome of 14 different villages of Kalimpong sub-division. The isolated pathogenic *Pythium* had white raised colony with compact aerial mycelium. Sporangia were observed on few branched hyphae, lobulate bud like outgrowth was observed and it has a growth rate of 8.15 mm per day (Table 2).

sugar alcohols (manitol, sorbitol, dulcitol) [Table 3b]. Thus, this was the first report of characterization of*Ralstonia solanacearum* ginger isolate from hill agro-climatic region of West Bengal and ginger *Ralstonia solanacearum* isolate belong to biovar III group.

Associated namatodes

Two associated nematodes have been identified as *Pratylenchus and Meloidogyne.* The morphological features of the isolated *Pratylenchus* sp. (both male and female) was 0.65 mm long having spear length of 17µ. The ratio between body length and width (a) was 36 (♂) & 31 (♀), body length and oesophagus (b) was 6 (both ♂ & ♀), body length and tail (c) was 23 (♂) & 22 (♀), and vulvular distance and total length of the body (v) was 50mm (♂) & 78 (♀). (Table 4) Pathogenecity of *Pratylenchus* spp. has been reported by Thapa *et al.* (2008).

Meloidogyne sp. was identified as another associated nematode. The morphological features of the *Meloidogyne* (female) was 0.7 mm long having spear length of 36µ. The total body length of larva was 0.5 mm. Here, it is very difficult to distinguish morphometric characters of the isolated larva because genital organs are not fully developed in larval stage. In case of larva, the ratio between body length and width (a) was 28, body length and oesophagus (b) was 7, body length and tail (c) was 9 and spear length was 29µ.

Association of different fungus, bacteria and nematodes has been found in present investigation and it can be concluded that the Rhizome Rot and Wilt disease of ginger in Darjeeling hills has a very complex etiology. Among two types of *Fusarium, Fusarium moniliforme* was found to be dominating in Kalimpong. The Gram negative rods, *Ralstonia solanacearum* Biovar-III, has been found to be associated with the disease. Nematodes like, *Pratylenchus and Meloidogyne* were present in the soil and infected plant parts. However, their role in disease development mechanisms needs to be studied furthermore.

REFERENCE

Thapa, A., Gurung, N., Sarma, Y. R., Bridge, J. and Jackson, G. V. H. 2008. Pathogenicity of Pratylenchus spp. On Ginger and Maize in the Indian Himalayas. *Indian Journal of Nematology* **38**: 107 – 109.