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Studies on Leaf Spot of Thankuni (*Centrella asiatica)* caused by *Alternaria* sp.

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In this paper, fixed plot survey symptoms of leaf spot of thankuni (*Centrella asiatica*), isolation of pathogen from *Centrella asiatica*, pathogenicity test of isolated pathogen for confirmation of disease, colony characters and growth of pathogen in different semi solid and liquid media and management of leaf spot of Thankuni under *in vitro* condition were made. The results of fixed plot survey showed that the highest leaf spot disease of Thankuni was recorded during February and July and lowest disease incidence was recorded during December and April. Studies of symptoms of leaf spot of Thankuni were recorded. The pathogen, *Alternaria* sp. was established through pathogenicity test. Morphometric characters and micrometric measurement of the pathogens were made. The antagonistic potential of three isolates each of *Trichoderma harzianum* and *Pseudomonas fluorescens* collected from laboratory of AICRP on Medicinal and Aromatic Plants and Betelvine centre were tested against the pathogen by Dual Culture Plate Technique. All the three isolates of *Trichoderma* were highly effective while two isolates of *Pseudomonas fluorescens* were highly effective and one isolate was moderately effective against *Alternaria* sp. under *in vitro* condition

Key words: *Centrella asiatica*, *Alternaria* sp., *Trichoderma*, *Pseudomonas fluorescens*

INTRODUCTION

In spite of the tremendous progress in the development of modern medicine, plants continue to be an important source of drugs throughout the world, particularly in the developing countries. According to Janardhanan (2002), even in highly developed country like USA, more than 25% of the prescriptions were found to contain one or more plant products. India is known for its various systems of traditional medicines (TM) that have been developed and practiced from the time immemorial. It is the home of 800 (of all) plant species on the earth, many of which had not been fully explored and cultivated. Cultivation of medicinal plants in West Bengal is yet to take a noble shape. Government of West Bengal has recommended some medicinal plants like Aswagandha, Sarpagandha, Senna, Tulsi etc for commercial cultivation in different zones. However, several biotic and abiotic factors limited the production of these crops. Several biotic factors like fungi, viruses, bacteria, phytoplasmas, nematodes and abiotic factors like deficiencies in soil, lack of proper irrigation, etc. are responsible for the maladies of medicinal plants.

The pathogenic diseases caused significant damage of the crops as well as reduced the quality of the produces and their market value. *Centrella asiatica* suffers from leaf spot caused by *Cercospora centellae*, Bacterial wilt disease caused by *Pseudomonas solanacearum* and the leaf spot disease caused by bacteria *Cochliobolus geniculatus* (Chowdhury, 2011). Other than the work conducted under A.I.C.R.P on Medicinal and Aromatic plants, very little work has been made on diseases of Thankuni (*Centrella asiatica*). In this present investigation, attempts has been made to study the leaf spot of *Centrella asiatica* caused by *Alternaria* sp.

MATERIALS AND METHODS

Fixed Plot Survey

Observations of the plots were done at monthly intervals starting from the month of April, 2010 to March, 2011 where the incidence and severity of the diseases which appeared on the medicinal plants were recorded. For per cent disease incidence total no. of leaves/stems infected in a plot were recorded and for per cent disease index, no. of leaves/stems infected per 10 plants in each plot were rated on a 0-4 scale, where 0= healthy leaves; 1= 1 - 6% leaf area infected; 2= 7 - 12% leaf area infected; 3= 13 - 25% leaf area infected; 4= above 25% leaf area infected. Per cent disease incidence and per cent disease index were calculated from the following formulae:

$$\text{Per cent Disease incidence} = \frac{\text{No. of leaves /stems infected in a plot}}{\text{Total no. of leaves/stems}} \times 100$$

and

$$\text{Per cent Disease Index} = \frac{\Sigma \text{ Numerical ratings}}{\text{No. of leaves} \times \text{Highest rating scale}} \times 100$$

Study of the disease symptoms

Disease conditions in the plants were recognized according to the symptoms produced by the pathogens. The maladies observed on the plants were recorded. The plants were carefully studied for any kinds of symptoms on the leaves, leaf petioles, stems, flowers and the rest of the above ground portions. Detailed descriptions of the symptoms are required for the establishment and diagnosis of the diseases later on.

Isolation of the pathogen
Collection of diseased specimen

The leaves which showed some spots or lesions were collected from the field for isolation of the fungi causing disease on them and brought to the laboratory.

Method of isolation

Isolation was carried out in a sterilized zone of the laminar air flow. The diseased specimens already washed with tap water were taken and with the help of a sterilized scissor, leaf was cut into small pieces which contained the diseased portion as well as the healthy tissue. The pieces were dipped in 0.1% $HgCl_2$ solution for 1 min. and were later rinsed three times with sterile distilled water. With the help of a sterilized forceps, each piece was placed aseptically on the solidified PDA / Water agar media on the sterilized plates depending upon the diseased specimen. About 3-4 such pieces were placed on each plate maintaining some distance from each other and the Petriplates were incubated at 28 ± 1°C. After 5 days, the growing fungus was examined under microscope for sporangial production.

The isolates were maintained on potato dextrose agar medium. All the isolates were preserved at 5 °C. Sub cultures were made at 15 days intervals. One set of isolates was preserved in liquid paraffin at 5 °C.

Pathogenicity test

Pathogenicity of isolated fungi were tested on potted plants by inoculating the leaves after removing all diseased leaves. The test was conducted with 3 replications and 5 plants per replication. Suitable control was maintained by spraying water.

A spore suspension (5 x 10^5 spores/ml) was prepared from 8 days old culture grown on potato dextrose agar medium was sprayed on leaves, with an all glass automizer and the whole set up was placed in the humid chamber. The lesion appeared after 2-3 days of inoculation were observed.

Morphometric character of the pathogen

The slides of the selected fungal cultures or colony were prepared in order to study the fungal morphology such as the characteristics of the hyphae,

conidiophores, spores, etc. for easy identification of the fungal species infecting a particular specimen. The prepared slides were observed under Phase-contrast microscope using ocular and stage micrometer.

Growth of Alternaria spp. in different liquid media

Alternaria sp. was grown in PDA, Czapek dox , Maize meal and Oat meal broth i.e. liquid media (50 mL in 250 mL Erlenmeyer flasks). All the flasks were inoculated with 6 mm mycelial disc grown on potato dextrose agar medium and incubated at 28 ± 1°C for 8 days. After 8 days dry weight of mycelial mats were recorded.

Dry weight determination

Coherent mycelium was removed from liquid medium. Washed thoroughly and dried on a preweighted filter paper at 65-70 °C for 48 hrs. The dried mycelial mat with filter paper was kept in a desiccator over anhydrous P_2O_5 and then weighed. Weighing was repeated till constant weight was obtained.

Radial growth of isolates of Alternaria spp. in different semisolid mediam

Alternaria spp. was grown in PDA, czapek dox , maize meal and oat meal media. Molten medium was poured into each sterile Petriplate and allowed to solidify. Small discs (6 mm) of the fungus mycelium was cut with a sterile disc cutter from margin of 7 days old culture grown in PDA and was transferred asceptically to the plates and incubated at 28 ± 1°C. Colony diameters were measured up to 8 days from 2nd day of inoculation.

Crop loss assessment in Centrella asiatica due to diseases

The experiment was conducted at 'C' Block farm, Kalyani using 12 plots of 3´ x 2´ with two treatments (6 plots each) i.e. application of Dithane M-45 @ 0.25% and Blitox @0.25% for six times at an interval of 15 days and control treatment (no fungicide).

Disease incidence and yield (no. of leaves) per sq. m were recorded in both the treatment. Avoidable Yield loss (Ayl) was recorded from the formula

$\frac{Yp - Yu}{Yu} \times 100$ where, Yp= Yield under protected condition
Yu= Yield under unprotected condition

In vitro management of leaf spot of Thankuni Effect of fungicides on hyphal growth of Alternaria sp.

The fungicidal solutions were prepared on the basis of active ingredients (ai) of the products and to determine the fungicidal effect on hyphal growth, poisoned food technique was followed using PDA as food base.

Screening of antagonist against Alternaria sp. Collection of antagonist from the soil

Three different isolates of *Trichoderma viride* and *Trichoderma harzianum* were collected from Department of Plant Pathology, Kalyani (Nadia) and three isolates of *Pseudomonas fluorescens* were collected from AICRP on Medicinal and Aromatic Plants and Betelvine. All the isolated were maintained on PDA slants at 5°C.

Antagonistic potential of Trichoderma isolate

The antagonistic properties of *Trichoderma* isolate was tested on PDA medium by Dual Culture Plate Technique. Five days old culture of *Alternaria* sp. was plated aseptically at the edge of Petri plates 2 days before the placement of isolates of *Trichoderma* sp. Paired cultures were observed upto 9 DAI. All the ratings were done after contacts between pathogens and antagonist using a modified Bell's (Bell *et al.,* 1982) scale (1-5) developed as follows:

Class I(R_1) – The antagonist completely overgrew the pathogen (100% overgrowth)

Class II (R_2) – The antagonist overgrew at least 2/3rd of pathogen surface (75% overgrowth)

Class III (R_3) – The antagonist colonized on half the growth of the pathogen (50% overgrowth)

Class IV (R_4) – The pathogen and antagonist locked at the point of contact; and

Class V (R_5) – The pathogen overgrew the mycoparasite

Antagonistic potential of Pseudomonas fluorescens isolates

The antagonistic properties of *Pseudomonas*

fluorescens isolates were tested on PDA medium by Dual Culture Plate Technique. Five days old culture of antagonist under study was inoculated by making long streak on the two sides of the plate keeping 1 cm away from the periphery aseptically containing PDA medium. At the centre of Petri plates, 2 days after the inoculation of antagonist, 5 days old culture of the fungi under study in PDA were placed aseptically in the centre of the palte. In control treatment, only 5 days old culture of the fungi under study in PDA was placed aseptically in the center of the Petri plate containing PDA medium. When full plate fungal growth in control treatment was noticed, the growth of the fungus in treated plate was recorded. The zone of inhibition between fungal growth and antagonist growth was also recorded. Per cent inhibition due to antagonist was recorded following the formula:

$$\text{Per cent Inhibition} = \frac{\text{Full growth of the test pathogen in control treatment - Growth of the test pathogen in treated treatment}}{\text{Full growth of the test pathogen in control treatment}} \times 100$$

RESULTS AND DISCUSSION

Fixed Plot Survey

The results (Table 1) showed that highest disease was recorded during February (PDI) and July (PDX). Lowest disease incidence was recorded during December (PDI) and April (PDX).

Symptoms of Leaf spot Disease of Thankuni *(Centella asiatica)*

The initial symptoms of the disease appeared as yellowing and discoloration of leaves. The spot starts from tip or margin of the leaves. Later the spots enlarge in size and turn dark brown in colour. It spread on whole leaf and affected tissues become dry. The older leaves become heavily affected than younger ones. The heavily affected leaves showed blighting. The disease causes heavy reduction in yield as the leaves are used for preparation of medicine or it is directly used as treatment of several diseases.

Colony characters of the pathogens

Visual observations of the colony characters were made after re-isolation from the inoculated plants with the pathogens (Table 2)

Table 1 : Fixed plot survey leaf spot of Thankuni

Month	% Disease Index (PDI)	% Disease Incidence (PDX)
April'10	13.78	15.0
May	14.94	26.08
June	16.22	24.24
July	16.24	30.41
August	-	-
Sept.	-	-
October	-	-
Nov.	-	-
Dec.	9.60	-
Jan'11	19.23	-
Feb.	22.94	-
March	-	-

Micrometric Measurements

Micrometric measurement of the pathogen were made after growing in PDA media and observed under the high power microscope (Table 2).

Table 2 : Colony character and the length and breadth of the spores

Colony Character of the pathogen	Length (µ)	Breadth (µ)
Blackish brown submerged mycelium. The back of the medium was black	27- 192	5-10

Cultural Characteristics of pathogens in different media

Characteristics of *Alternaria* spp. was studied in four different media. The general cultural features of the pathogens are presented in Table 3.

Radial growth of Alternaria sp. *in different semi solid media*

The fungus was allowed to grow in four different media such as Potato Dextrose Agar Media (PDA), Czepek dox media (CZA), Maize meal agar media (MMA) and Oat meal agar media (OMA). Data was taken after every 48 hrs until full growth of the fungus takes place. The results (Table 4) showed that highest growth of *Alternaria* sp. was recorded CZA media where as the lowest growth of was recorded in MMA media. The results thus revealed that CZA media is the best for the growth of the pathogen.

Growth of Alternaria sp. *in different liquid medium*

The test pathogens were grown in four liquid me-

dia (Potato Dextrose, Czapek dox, Maize Meal and Oat Meal broth) for 15 days and dry weight was determined. The results (Table 3) revealed that highest dry weight was recorded in Czapek Dox broth and lowest dry weight was recorded in Oat meal broth.

Table 3 : Cultural Characteristics and radial growth of the pathogen in different media

Growth of Pathogen in different media	Potato dextrose Agar Medium	Czapek Dox Agar Medium	Maize Meal Agar Medium	Oat Meal Agar Medium
	Light blackish mycelia growth, submerged mycelia with cottony growth takes place. growth of the fungus is slow. Full plate growth of the fungus takes place in.	Olivaceous green with whitish mycelia growth takes place. Mycelium is not well distributed in the plate, somewhere fluffy and somewhere submerged. Full plate growth of the fungus takes place in.	Brownish growth of mycelia occurs. Submerged mycelia well distributed through whole plate. Full plate growth of the fungus takes place in.	Black colour mycelia with brownish growth take place. Submerged mycelia with fluffy growth at the centre, black colour at periphery and brown colour at the centre of the plate. Full plate growth of the fungus takes place in.
Radial growth in different media	84.5mm	90mm	83mm	87mm
Dry wt. (gm) in different media	1.8gm	2.3gm	1.5gm	1.2gm

Crop loss assessment in Centella asiatica due to diseases

The experiment was conducted at 'C' Block farm, Kalyani using 12 plots of 3´ x 2´ with two treatments (6 plots each) i.e. application of Dithane M-45 @ 0.25% and Blitox @0.25% for six times at an interval of 15 days and control treatment (no fungicide). The results revealed that in treated plots disease incidence was 10.42-14.90% and in untreated plots disease incidence was 17.60-23.10%. The percent disease control was 28.47-46.87%. The avoidable yield loss was calculated and it was recorded 17.30-34.47%. From the results it can be concluded that the avoidable yield loss is not directly related with the incidence of disease as where maximum disease control was noticed, the avoidable yield loss was recorded low (Table 4).

Effect of Fungicides on Mycelial Growth of Alternaria sp.

Per cent inhibition in mycelial growth of *Alternaria* sp. was recorded by poisoned food technique using different concentrations of test fungicides. Through log-probit analysis ED_{50} values of different fungicides towards *Alternaria* sp. were determined (Table 5).

The results showed (Table 5) that among the fungicides tested the lowest and highest ED_{50} values were recorded in Carbendazim and Copper oxychloride (Blitox) respectively.

Table 4 : Crop loss assessment in *Centella asiatica* due to leaf spot disease caused by A*lternaria* sp

Plot no.	Crop loss assesment in Thankuni (*Centella asiatica*)due to Leaf spot disease cause d by *Alternaria* sp					
	Treated plot(per cent disease index)	Untreated plot (per cent disease index)	Per cent disease control	Yield (no. of leaves per sq.m) in treated plot	Yield (no. of leaves per sq.m) in untreated plot	Avoidable Yield loss
One	11.65	21.90	46.80	2373	1555	34.47
Two	12.15	21.80	44.26	2115	1749	17.30
Three	14.90	23.10	35.49	2578	1932	25.05
Four	12.22	17.10	28.47	1942	1577	18.79
Five	10.42	17.60	40.79	2174	1738	20.05
Six	10.97	20.65	46.87	2164	1776	17.92

Screening of Trichoderma and Pseudomonas fluoroscens isolates against Alternaria sp

The results (Table 6) showed that all the three isolates of *Trichoderma* were highly antagonistic (R_1) against *Alternaria* sp. and isolate P_1 of *Pseudomonas fluoroscens* was highly antagonistic against *Alternaria* sp. as higher per cent inhibition and zone of inhibition was recorded against the pathogen whereas, isolate P_3 of *Pseudomonas fluoroscens* was weakly antagonistic against the pathogen as revealed from per cent inhibition and zone of inhibition.

have to be collected from the medicinal plant garden. The bio-agents and safer fungicides should be tested under field conditions before giving recommendation to the farmers.

Bell's ranking: R_1 = The antagonist completely over grew the pathogen and cover the entire medium surface; R_2 = The antagonist over grew at least $^2/_3$ of the pathogen's surface; R_3 = The antagonist colonized on ½ of the growth of pathogen; R_4 = The pathogen and antagonist locked at the point of contact and R_5 = The pathogen over grew the mycoparasite.

Table 5 : ED_{50} value of different fungicides towards mycelial growth of *Alternaria* sp.

Name of the fungicide	Trade name	Chemical name	ED_{50} value in ppm
Copper oxychloride 50WP	Blitox	Copper oxychloride preparation	223.87
Carbendazim	Bavistin	Methyl-2-benzimidazole carbamate	3.34
Mancozeb	Dithane M-45	Manganous ethylene bisdithiocarbamate	44.66

* Average of three replications

Table 6 : Screening of *Trichoderma* and *Pseudomonas fluorescens* isolates against *Alternaria* sp.

Pathogens	Isolates of *Trichoderma* sp.	Point of contact (day)	Bell's ranking (modified)
Alternaria sp.	T_1	3	R1
	T_2	3	R1
	T_3	3	R1
	Isolates of *Pseudomonas fluoroscens*	Per cent inhibition	Zone of inhibition (mm)
	P1	49	13 mm
	P2	48	11.5 mm
	P3	21	9 mm

From the results it could be concluded that leaf spot of *Centrella asiatica* caused by *Alternaria* sp. causes 17-34 per cent losses in yield. There is a scope for using biocontrol agents like *Trichoderma* and *Pseudomonas fluorescens* and some safer fungicides to check leaf spot disease of *Centrella asiatica*. However, the bio-agents to be tested will

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