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SOLANKI SARKAR, B. DASGUPTA AND RAJIB MANDI

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Department of Botany,
University of Calcutta,
Kolkata 700 019, India

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Studies on Leaf blight disease of pipul (*Piper longum*) with special emphasis on management of the disease

SOLANKI SARKAR, B. DASGUPTA* AND RAJIB MANDI
Department of Plant Pathology, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur 741252, West Bengal

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India is known for its various systems of medicines (TM) that had been developed and practiced from the time immemorial. Among the cultivable medicinal plants in India particularly in West Bengal, Pipul (*Piper longum*) is one of the most important crop which suffers from leaf blight disease caused by *Colletotrichum gloeosporioides.* The present investigation was carried out were fixed plot survey; symptoms, isolation of pathogen, pathogenicity test of isolated pathogen for confirmation of disease, colony characters and growth of pathogens in different semi solid and liquid media and management of the disease using safer fungicides and biocontrol agents. The results of fixed plot survey showed that the highest blight disease of Pipul was recorded during March 2013, while lowest disease was recorded during September 2012. The symptoms recorded were dark chocolate, irregular and angular spots surrounded by yellow halo which extended from the margin and any part of the leaf. No spots were found on the younger leaves. The pathogenecty test confirmed the above pathogen causing disease. Micrometric measurement of the pathogen was made and the size of the spores were 5.6 – 8.5 x 0.55 – 1.38 µ. The pathogen was allowed to grow in different liquid and semi-solid media. Highest growth and dry weight of was observed in PDA media and broth. The antagonistic potential of the bioagents were studied by Dual Culture Plate Technique showed that *Trichoderma* isolates T2 and T3 were highly antagonistic to *C. gloeosporioides.* Poisoned food technique was used based on different concentrations of test fungicides. The result showed that ED_{50} value of Blitox, Bavistin and Mancozeb were 354.81ppm, 446.68ppm and <50ppm respectively. Field trial by using safer fungicides and biocontrol agents showed that highest disease (56.43%) was recorded in control treatment and application of Bavistin recorded the lowest percent disease incidence of blight of Pipul caused by *Colletotrichum gloeosporioides* (23.04%) and recorded 59.17% control of the disease over control.

Key words: Pipul, *Colletotrichum gloeosporiodes*, Bavistin, *Trichoderma*

INTRODUCTION

India has a rich culture of medicinal herbs and spices, which includes about more than 2000 species and has a vast geographical area with high potential abilities for Ayurvedic, Unani, Siddha traditional medicines but only very few have been studied chemically and pharmacologically for their potential medicinal value. Several biotic factors like fungi, viruses, bacteria, phytoplasmas, nematodes and abiotic factors like deficiencies in soil,

*Corresponding author : dasgupta.basudeb824@gmail.com

lack of proper irrigation, etc. are responsible for the maladies of medicinal plants. Pipul (*Piper longum*) suffers from root rot complex and *Colletotrichum* leaf blight (Ingle *et al,* 2011), leaf spot disease caused by *Botrytis cinerea* (Shikha and Harsh, 2012), foot rot caused by *Phytophthora parasitica [Phytophthora nicotianae* var. *parasitica*]. Very little work other than the work conducted under AICRP on Medicinal and Aromatic plants has been made on diseases of *Piper longum.* In this present investigation, attempts have been made to study the leaf blight disease of Pipul caused by *Colletotrichum gloeosporioides* with special emphasis on management of the disease.

MATERIALS AND METHODS

Fixed plot survey

Observations of the plots were done at 15 days intervals starting from the month of August, 2012 to June, 2013 where the incidence and severity of the diseases which appeared on pipul was recorded. For percent disease incidence total no. of leaves/stems infected in a plot were recorded and for percent disease index, no. of leaves infected per 10 plants in each plot were rated on a 0-5 scale, where 0= healthy leaves; 1= 1 - 10% leaf area infected; 2= 11 - 20% leaf area infected; 3= 21 - 40% leaf area infected; 4= 41-60% leaf area infected and 5= above 61% leaf area infected. Percent disease incidence and percent disease index were calculated from the following formulae :

$$\text{Percent disease incidence} = \frac{\text{No. of infected leaves per plant}}{\text{Total no. of leaves per plant}} \times 100$$

$$\text{Percent disease incidence} = \frac{\sum \text{Numerical ratings}}{\text{Total No. of units observed} \times \text{maximum rating}} \times 100$$

Study of the disease symptom

Disease conditions in the plants were recognized according to the symptoms produced by the pathogens. The plants were carefully studied and symptoms observed on the plants were recorded.

Isolation of the pathogen
Collection of disease specimen

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

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, small pieces of the leaf were cut into small pieces which contained the diseased portion as well as the healthy tissue. The pieces were dipped in $HgCl_2$ solution for 1 min. and were later rinsed three times with sterile distilled water under aseptic condition. With the help of a sterilized forceps, each piece was placed aseptically on the solidified PDA 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 $\pm$ 1^0C. After 5 days, the growing fungus was examined under micro-scope for sporulation and identification of the pathogen.

The isolates were maintained on potato dextrose agar medium. All the isolates were preserved at 5^0C. Sub cultures were made at 15 days intervals.

Pathogenicity test

Pathogenicity of isolated fungi was 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.

Confirmation of pathogens

After the appearance of disease symptoms, the diseased leaves were collected and again re-isolated the pathogens to compare with the previous isolated pathogens and to get confirm about the disease causing pathogens.

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 and 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.

Colony characters and radial growth of Colletotrichum gloeosporiodes in different semisolid media

Colletotrichum gloeosporiodes 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. Different changes of fungal colony in different media were recorded every day up to 10 days. Colony diameters were measured up to 10 days from 2nd day of inoculation.

Growth of Colletotrichum gloeosporiodes in different liquid media

Colletotrichum gloeosporiodes 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 pre weighted 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.

In vitro management of leaf blight disease of Pipul
Effect of fungicides on hyphal growth of Colletotrichum gloeosporioides

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. After autoclaving and cooling (45–50 ^{0}C) different concentration of fungicides as per treatment were incorporated/mixed into the molten Potato Dextrose agar media. This sterile molten media containing fungicide was poured aseptically into sterile petriplates. In control only molten media without fungicides was poured. Each treatment as well as control was replicated thrice. Each plate was aseptically inoculated with mycelial disc and incubated at 28 ± 1 ^{0}C. The colony diameter of the fungus was measured when in control full plate growth was observed.

Per cent inhibition of growth was calculated by the formula :

$$\% \text{ growth inhibition} = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100$$

ED_{50} values of different fungicides towards inhibition of hyphal growth was determined by log probit analysis (Log of Concentration of fungicide and probit value of hyphal growth inhibition).

Screening of antagonist against Colletotrichum gloeosporioides
Antagonistic potential of Trichoderma isolate

The antagonistic properties of *Trichoderma* isolates which were collected from AICRP on Medicinal and Aromatic plants and Betelvine was tested on PDA medium by Dual Culture Plate Technique. 5 days old culture of the fungi under study were plated aseptically at the edge of petri plates 2 days before the placement of *Trichoderma sp*. Paired cultures were observed for a total of 9 days before being discarded. 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 2/3 rd 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.
Class V (R_5) – The pathogen overgrew the mycoparasite.

Field trial

A field trail was conducted at 'C' Block farm, Kalyani. The chemicals and bioagents were sprayed at 15 days interval for three times.

To prepare a spore suspension of *Trichoderma* spp, eight days old culture plates were used, which were grown on PDA at 28±1ºC. The plates were rinsed by brush with sterilized distilled water. The suspension was then filtered by muslin cloth to separate the spores from the mycelia. The concentration was adjusted to 3.7×10^8 spores/ ml with the help of haemocytometer.
The treatments were:

Tr_1 = Spraying of Blitox @0.25%
Tr_2 = Spraying of Carbendazim @0.1%
Tr_3 = Spraying of Mancozeb @0.25%
Tr_4 = Spraying of *Trichoderma-1* @ 3.7×10^8 cfu per ml
Tr_5 = Spraying of *Trichoderma*-2 @ 3.7×10^8 cfu per ml
Tr_6 = Spraying of *Trichoderma*-3@ 3.7×10^8 cfu per ml
Tr_7 = Control

Spraying was done as per treatment schedule. Before starting the experiment all the infected leaves in treatment rows were removed. Last application of chemicals is done in the month of July. Results were recorded by counting the number of infected and healthy leaves in treatment rows. Per cent disease incidence (PDI) was calculated as mentioned earlier. The results obtained were subject to analysis of variance.

RESULTS AND DISCUSSION

Fixed plot survey

The results (Table 1) revealed that the highest percent disease incidence and percent disease index were recorded during March,2013 (65.38%) and June,2013 (69.33%) respectively and lowest percent disease incidence and percent disease index were recorded during September,2012 (28.62% and 20.0%) respectively. During the survey period no disease was recorded during August, 2012.

Symptoms of Leaf blight of Piper longum

Dark chocolate, irregular and angular spots occurred on the leaves. The spots were surrounded by yellow halo which extend from the margin and any part of the leaf. Later the spots enlarge in size and 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.

Table 1 : Fixed plot survey of diseases

Months	% Disease Incidence.	% Disease Index
Aug,12	_	_
Sept,12	28.62	20
Oct,12	38.31	28
Nov,12	45.81	36
Dec,12	47.39	42
Jan,13	55.16	48.67
Feb,13	61.44	53.33
Mar,13	65.38	58.66
April,13	61.47	62.66
May,13	59.70	66
June,13	59.41	69.33

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).

Micrometric measurements

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

Growth of pathogens in different semi solid media

The fungi was allowed to grow in four different media such as Potato Dextrose Agar media (PDA), Czepek dox media (CZA), Oat meal agar media (OMA), Maize meal agar media (MMA). Data was taken for ten days. The results showed that the highest growth of *Colletotrichum gloeosporioides* was observed in PDA (9.0 cm) and lowest growth in maize meal media (4.8) (Table 3).

Growth of pathogens in different liquid media:

The fungi was grown in four different liquid media

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

Colony character of the pathogen	Length of spores (μ)	Breadth of spores (μ)
Dark gray – white fluffy colony; back of the media was completely black	5.60 – 8.50	0.55-1.38

Table 3 : Colony characteristics and growth of the pathogen in different media

Growth of the pathogen in different media	Potato Dextrose agar medium	Czepek dox agar medium	Oatmeal agar medium	Maize meal agar medium
	At first milkish white mycelia growth started on media. Acervuli formation started at the whole periphery of the plate and after some day covered the plate	Whitish submerged mycelia growth started. Black granulation (acervuli) are covered the whole plate but it was not so dense	Whitish mycelium growth on which black dense doted acervuli are present	Whitish mycelia growth is formed. Black acervuli are scattered on the mycelia growth but it not so dense.
Radial growth in different media (cm.)	9.00	7.60	8.10	4.80
Dry wt. (gm) in different media	1.47	1.19	1.31	1.31

Table 4 : ED_{50} value of different fungicides towards mycelial growth of *Colletotrichum gloeosporioides*

Name of the fungicide	Trade name	Chemical name	ED_{50} value in ppm
Copper oxychloride 50WP	Blitox	Copper oxychloride preparation	354.81
Carbendazim 50WP	Bavistin	Methyl-2-benzimidazole carbamate	446.68
Mancozeb 75WP	Dithane M-45	Manganous bisdithiocarbamate	ethylene <50

Table 5 : Screening of *Trichoderma* isolates against *Colletotrichum gloeosporioides*

Pathogens	Isolate of *Trichoderma sp*	Point of contact (day)	Bell's ranking
Colletotrichum gloeosporioides	T_1	3	R2
	T_2	3	R1
	T_3	3	R1

Table 6 : Management of leaf blight of *Piper longum* using safer fungicides and biocontrol agents

Treatments	Percent Disease Incidence	Percent Disease Control
1. Spraying of Blitox (0.25%)	27.33 (31.50)*	44.18
2. Spraying of Carbendazim (0.1%)	15.34 (23.04)	59.17
3. Spraying of Mancozeb (0.25%)	16.43 (23.90)	57.65
4. Spraying of *Trichoderma*-1	32.58 (34.79)	38.35
5. Spraying of *Trichoderma*-2	32.80 (31.92)	43.43
6. Spraying of *Trichoderma*-3	27.26 (31.46)	44.25
7. Control	69.47 (56.43)	
SEm±	(4.59)	
CV%	(9.51)	

* Figures in parentheses are the angular transformed values of percent disease incidence

such as Potato Dextrose, Czepek dox, , Oat meal, Maize meal broth for 15 days. After that dry weight was determined. The results (Table 3) revealed that highest dry weight of Colletotrichum gloeosporioides was observed in PDA media.

Effect of fungicides on mycelial growth of Colletotrichum gloeosporioides

Per cent inhibition in mycelial growth of *Colletotrichum gloeosporioides* was recorded by poisoned food technique using different concentrations of test fungicides. Through log-probit analysis ED50 values of different fungicides towards Alternaria sp. were determined (Table 4).

The results showed (Table 4) that among the fungicides tested the lowest and highest ED50 values were recorded in Carbendazim and Copper oxychloride (Blitox) respectively.

Antagonistic study of Trichoderma against pathogens

The three isolates of *Trichoderma* were tested against *Colletotrichum gloeosporioides* by dual plate technique and rating of antagonism was recorded according to the modified Bell's ranking (Bell *et al,*1983).

The results (Table 5) revealed that isolates T_2 and T_3 were highly antagonistic to *C. gloeosporioides* where as T_1 was moderately antagonistic

Management of leaf blight diseases of Pipul (Piper longum) by using safer fungicide and bio control agents

The results revealed that the lowest disease (23.04%) was recorded in treatment where spraying of carbendazim (0.1%) were made (T_2) and it was statistically at par with all the treatments except where isolate Trichoderma-1 was sprayed (T_4) and control treatment. Highest disease (56.43%) was recorded in control treatment (Table no.6).

The results thus obtained revealed that application of Bavistin (0.1%) recorded highest leaf blight disease control. The results also revealed that application of fdungicides were better than application of biocontrol agents. The results obtained are in consonance with the results obtained by Choudhury (2011) and Choudhury *et al*, (2015) where application of Carbendazim and Mancozeb recorded lowest disease incidence for the management leaf spot of Thankuni and target leaf spot of Sarpagandha in comparison with the biocontrol agents. However, for the management of any medicinal plant diseases, it will be better if we can control the disease by using biocontrol agents to reduce the toxic hazards of fungicides on human beings. The results thus obtained needs further investigation before being recommendation to the farmers.

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