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BIOCHEMICAL CHARACTERIZATION OF PHYLLOSPHERIC ISOLATES AGAINST PHOMOPSIS BLIGHT OF EGG PLANT (*SOLANUM MELONGENA* L.) CAUSED BY *PHOMOPSIS VEXANS*

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Abstract

Fruit rot of brinjal caused by *Phomopsis vexans* is an important disease of eggplant inflicting heavy losses. The present study was carried out in the laboratory and poly house of Department of Mycology and Plant Pathology, B.H.U., Varanasi (U.P.), India to test the biochemical characterization of phyllospheric isolates against phomopsis blight of egg plant (*Solanum melongena* L.) caused by *Phomopsis vexans.* Maximum inhibition of 75.5% was recorded by fungal isolate F4 that covered $3/4^{th}$ of the pathogen colony followed by F1 and F5 isolates (73.3%). Maximum zone of inhibition was due to diffusible metabolite secreted by them. All the fungal isolates were found positive for the amylolytic and lipolytic assay while, it was found negative in proteolytic assay. F1 and F4 showed high Indole acetic acid (IAA) production, when compared to control with the amount being 201.46 and 66.53µg/ml, respectively while F1 and F2 recorded the highest activity of phosphate solubilization with values 1432.91 and 1875.26 µg/ml, respectively. Hence, F4 isolate was considered the best against phomopsis blight.

Key words : Phyllospheric isolates, *Solanum melongena* L., *Phomopsis vexans*, Indole acetic acid (IAA), healthy tissues.

Introduction

Brinjal or egg plant (*Solanum melongena* L.) belonging to the family Solanaceae is the second major vegetable crop next to potato in India. Total production of brinjal is about 32 million tonnes in the world wherein India is world's second largest producer after China. However, the crop is known to suffer from many diseases and among them phomopsis blight caused by *Phomopsis vexans* (Kemble, 2010) has been treated as one of the major constraints to egg plant cultivation in the country. It can also be referred to as stem blight or canker, leaf blight or spot and fruit rot. *Phomopsis vexans* reduces yield and marketable value of the crop nearly 20-30% (Kaur *et al*., 1985). It is mainly spread by spores, which are usually released from a fungal fruiting body (pycnidia) and dispersed by splashing rain, insects and contaminated equipment. Spores germinate rapidly when free moisture is present on leaves, stems or fruits. The fungus survives between eggplant crops on and in crop debris, seeds and soil (Howard and David, 2007). The disease is difficult to manage because the slippery skin of the fruit does not allow good adhesion of sprays (Mark, 2010).

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Materials and Methods

The present investigation was carried out in the laboratory and poly house of Department of Mycology and Plant Pathology, Institute of Agricultural Sciences (I.Ag.Sc), Banaras Hindu University (B.H.U.), Varanasi (U.P.), India; with the aim to find out an efficient indigenous biocontrol agent from the phyllosphere of agronomically important crops for the management of blight of brinjal caused by *Phomopsis vexans* (Lib.) de Bary.

Isolation of pathogen

The pathogen *P. vexans* was isolated on PDA medium from the lesions of infected fruit of egg plant. A small portion of diseased tissue along with a portion of adjacent healthy tissue were cut into small pieces (3 to 5 mm in length) and then surface sterilized with 0.1% $HgCl_2$ for 30 seconds. The pieces were then rinsed thrice with sterilized distilled water. Sterilized and rinsed pieces were inoculated aseptically on sterilized petriplates containing PDA medium. The inoculated petriplates were incubated at 20 to 25^{0}C for five to six days. When the fungal colony was fully developed, it was sub cultured on fresh petriplate containing PDA medium to obtain its pure culture. All

the isolates were subjected to pathogenicity test on 30 days' old egg plants under green house conditions (Mathur and Kongsdal, 2003). It was followed by Dual culture plate assay.

Biochemical characterization of fungal isolates

a) Amylolytic activity

To test the degradation of the activity of amylase enzyme nutrient agar or PDA amended with 0.2% soluble starch was used. The pH of medium was 7.0. The phyllospheric fungal isolates were inoculated on the medium. After 3-4 days of incubation, the plates were flooded with iodine solution. A clear yellow zone around the colony confirms the activity of amylase enzyme.

b) Lipolytic activity

For lipase enzyme test, firstly lipase media containing tween 20 was prepared and was poured in sterilized petriplates. The fungal isolates were inoculated in the centre of the plates and were incubated at 28±2^{0}C for 2-3 days. After that the plates were observed carefully for yellow crystal formation, which indicated positive lipase activity.

c) Pectolytic activity

For testing pectolytic activity, we used the medium as described by Hankin *et al*. One litre of the medium contained 500 ml of mineral solution, 1 gm yeast extract, 15 gm of agar, 5 gm of pectin (citrus or apple) and 500 ml of distilled water. The pH of medium was kept 7.0. After sterilization, the media was poured in sterilized petriplates. A 5 mm mycelia disc of fungal isolates was inoculated on centre of plate after solidification of the medium. The plates were incubated at 28±1 ^{0}C for 2 days. After 2 days, when colony growth of isolates was seen, then the plates were flooded with 1% aqueous solution of Cetyl trimethyl- ammonium bromide (CTAB). Transparent circle around the mycelium seen was taken as the positive indication of pectinase activity.

d) Proteolytic activity

For protease test PDA media was prepared and poured in the sterilized petri plates. 8% solution of gelatin in water was sterilized separately and added to the medium at the rate of 5 ml per 100 ml of medium. pH was maintained at 6. The test microbes were inoculated in the centre of the plates as 5 mm mycelial disc and incubated at 28±1^0 C for 2-3 days. The plates were then flooded with saturated solution of ammonium sulphate. Clear zone around the mycelia colony indicated positive test for protease activity.

e) Indole Acetic Acid (IAA) production assay

IAA media was prepared by suspending 0.2gm tryptophan in 1000ml PDB. 10-15ml media were autoclaved in separate glass vials. Spores of the fungal isolates were inoculated in vials and were incubated at 200 rpm for 7 days. Post incubation, the culture was filtered and the filtrate was used for the quantitative estimations of IAA produced. To 1 ml filtrate, 2 ml of Salkowski's reagent was added and was incubated for 20 min at room temperature. A control was also maintained in which instead of culture filtrate, sterile distilled water was taken with Salkowski's reagent. Absorbance was measured at 535nm in a spectrophotometer. The values were calculated on the basis of standard curve formed by using various concentrations of IAA.

f) Phosphate solubilization assay

Specific media for phosphate solubilization that is NBRI-BPB was prepared. Media was sterilized separately in glass vials and was inoculated with spores of each isolate separately. Inoculated vials were incubated at 28ºC at 250 rpm for 3-4 days. The culture was filtered and the filtrate was used for estimating the amount of phosphate solubilised. 50 µl filtrate was taken and to it, 500 µl ammonium molybdate solution was added followed by 13 µl $SnCl_2$ and 2.5 ml distilled water. Blue colour developed was measured colorimetrically at 600 nm. Also, the pH of the medium was recorded, post incubation.

Results and Discussion

Antagonistic effect of all fungal isolates was studied *in vitro* against *Phomopsis vexans* in PDA medium by dual culture methods (table 1). Out of 6 fungal isolates, 4 were found to be effective in checking the growth of the pathogen. Maximum inhibition of 75.5% was recorded by fungal isolate F4 that covered 3/4th of the pathogen colony followed by F1 and F5 isolates (73.3%) that completely over grew the pathogen colony. Sharma *et al*. (1999) reported that inhibitory activity of *Trichoderma* might be due to the diffusible metabolite secreted by them, since pathogen parasite relationship revealed hyphal breakage of pathogen.

Amylolytic activity

The isolates of phyllospheric fungus were grown in medium, which was amended with starch to access the amylolytic activity. All the isolates were found to give positive results for the assay by forming a yellow zone around the colony, when the plates were flooded with iodine solution signifying the degradation of starch in the given areas by the action of amylase enzyme (table 2).

Table 1 : Dual culture of pathogen and phyllospheric fungal isolates.

Isolate name	DOP (cm)	DOA (cm)	MY/A	Percent Inhibition (%)	Extent of antagonist's growth over pathogen colony
F1	1.2	4.0	MY	73.3	Complete
F2	1.8	3.5	MY	60	Complete
F3	1.9	3.5	MY	57.7	1/3
F4	1.1	4.1	MY	75.5	3/4
F5	1.2	4.1	MY	73.3	Complete
F6	1.7	3.5	MY	62.2	1/2

*DOP- Diameter of pathogen, DOA- Diameter of antagonist.

Table 2 : Solid Plate Enzymatic assays of phyllospheric fungal isolates.

Sl. No.	Isolate name	Amylolytic assay	Lipolytic assay	Pectolytic assay	Proteolytic assay
1.	F1	+	++	-	-
2.	F2	++	+	+	-
3.	F3	++	++	-	-
4.	F4	++	++	+	-
5.	F5	+	+	+	-
6.	F6	++	++	+	-

Table 3 : IAA test for phyllospheric isolates.

Sl. No.	Isolate name	O.D. (535nm)	IAA (µg/ml)
1.	Control	0.154	20.53
2.	F1	1.511	201.46
3.	F2	0.195	25.99
4.	F3	0.177	23.59
5.	F4	0.499	66.53
6.	F5	0.252	30.93
7.	F6	0.181	24.13

Lipolytic activity

All fungal isolates gave positive result. Out of 6 isolates of fungi F1, F3 and F4 isolates have shown excellent result. The antagonists showed deposition of yellow colored crystal of calcium salt due to degradation of Tween 20 by the action of lipase enzyme (table 2).

Pectolytic activity

All the fungal isolates were grown on media amended with pectin and were analyzed by post incubation step for the activity of pectinase enzyme. All the isolates except F1 and F3 showed clear zone around the mycelium when the plates were flooded with 1% aqueous solution of hexadocyl trimethyl-ammonium bromide (CTAB) showing positive result for pectinase enzyme (table 2).

Table 4 : Phosphate solubilization assay of phyllospheric fungi.

Sl. No.	Isolate name	O.D. (600nm)	Amount of phosphate solubilised (µg/ml)	pH of the medium post incubation
1.	Control	0.158	371.76	6.6
2.	F1	0.609	1432.92	4.8
3.	F2	0.797	1875.26	4.0
4.	F3	0.154	362.35	5.4
5.	F4	0.422	992.92	5.5
6.	F5	0.179	421.17	5.8
7.	F6	0.272	639.99	6.3

Proteolytic activity

All the fungal isolates were grown on medium, which had gelatin as a source of protein for analyzing the proteolytic activity of the isolates. Post incubation, when the plates were flooded with saturated solution of ammonium sulphate all the isolates showed negative result forming no clear zone around the mycelial colony (table 2).

Indole Acetic Acid (IAA) production assay

All fungal isolates were inoculated for seven days in PDB medium containing tryptophan (100 µg/ml).

Colorimetric estimation of IAA production by the isolates was estimated by adding Salkowaski reagent to the culture filtrate. Out of 6 fungal isolates F1 and F4 showed high IAA production when compared to control with the amount being 201.46 and 66.53 µg/ml, respectively. The amount of IAA produced was calculated by the standard curve formed by using various concentration of IAA (table 3).

Phosphate solubilization assay

All the isolates were grown in NBRI-BPB medium for 7 days and the culture filtrate was taken for the quantitative estimation for phosphate solubilization at 600nm. Change in the pH of the medium was also recorded post incubation. Among 6 isolates F1 and F2 recorded highest activity of phosphate solubilization with values 1432.91 and 1875.26 µg/ml, respectively. Drop in the pH from 6.6 in the control to 4.0 was recorded signifying the amount of organic acid formed as a result of solubilisation of inorganic phosphate. The change in the color of the medium could also be observed as a qualitative estimation for phosphate solubilisation (table 4).

Conclusion

Zone of maximum inhibition is recorded in the isolate F4. Hence, F4 is considered as best among all the six phyllosphere isolates on the basis of biochemical characterization against phomopsis blight caused by *Phomopsis vexans*.

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