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Management Of Phytophthora Capsici ,Causative Fungi Of Foot Rot Of Pepper By Endophytes Isolated From Azadirachta Indica As Promising Biocontrol Agent

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Abstract

Fungicides and organophosphate insecticides are the current recommendations, being widely used to combat Phytophthora and nematodes in black pepper. Even though most effective, they are polluting the environment and residue remains with the product that are hazardous. During the past two decades researchers pursued options like host resistance, organic amendments and biocontrol agents to chalk out an integrated pest management strategy with varied success rates. Some of the prominent recommendations emanated in India are the combined application of potassium phosphonate, neem oil cake and biocontrol agents like Trichoderma harzianum, T. viride, Pseudomonas fluorescens and Pochonia chlamydosporia. PGPR improves the plant growth by preventing the proliferation of phytopathogens and thereby support plant growth. Endophytes are beneficial microbes that grow within the plants without showing any visible symptoms. Being ubiquitous in plant tissues, they can be isolated from leaves, stem, roots, seeds, fruits and flowers. The direct effect of endophytes in promoting plant growth are thought to include phytohormone production, asymbiotic nitrogen fixation, solubilization of inorganic phosphate and mineralization of organic phosphate/other nutrients.

Keywords: Azadirachta indica, Phytophthora capsici, Endophytes, PGPR, Biochemical tests, Piper nigrum.

Introduction:

Black pepper (Piper nigrum L.), sometimes referred to 'black gold'(Nair and Sarma, 2004), is a high value export crop for countries such as India, Indonesia, Malaysia, Thailand, Sri Lanka, Brazil, China and Vietnam (Nair and Sarma, 2004). The production of black pepperoffers a good source of cash income for farmers in developing countries and the rate of growth in production area and yield has increased significantly, from 1% to 17.6% worldwide, over the

past few years (Peter, 2000). Phytophthora foot rot, also known as 'quick wilt', of black pepper has been reported throughout the world where the crop is grown (Anandaraj and Sarma, 2003). According to Sarma et al. (1988), use of the term 'wilt' in black pepper is loose and does not reflect true vascular wilts in the strict phytopathological sense. Quick wilt is used because of the external symptoms of disease when the collar of vine was infected and killed within a few days. The term 'Phytophthora foot rot' was given precedence over 'quick wilt' or 'foot rot' of black pepper at the International Community Workshop on Joint Research for the Control of Black Pepper Diseases in 1988 at Goa, India (Nair and Sarma, 2004).

Field survey plays an important role in assessing the relation of pathogen with weather condition depending upon soil and variety. Survey suggests the role of pathogens in the yield loss. Pathogens causes wilt, seedling infections, foot rot, root-rot, leaf spot, rust, in which pathogens plays an important role in disseminating the diseases (Anandaraj and Sarma, 2003). Molecular markers are new genetic tools with potential to enhance selection efficiency and are advantageous, since these have no effect in themselves on the phenotype. The number of availability of molecular markers is enormous and also has the advantage that the heterozygote of these can be identified.

Endophytic fungi have been reported from various plant species, which contribute to the diversity of microorganisms in innate environment and produce various bioactive compounds and novel metabolites (Strobel et al., 2004; Sun et al., 2008). The composition of the fungal community usually differs between host species (Frommel, 1991), among the geographically separated individuals of the same host species (Jackson and Taylor, 1996), and also within the various tissue or organs of a host plant. The endophytic fungal community confirmed host specificity at species level but this specificity could be influenced by environmental conditions (Klessig and Malamy, 1994), also known as 'spatial heterogeneity' or 'geographic variation' (Arnold, 2007). Differences of endophytic fungal assemblages in different tissue types have been reported in the same plant species, or even in different tissues of an individual plant, which is a reflection of tissue specificity.

MATERIALS AND METHODS

Bark and leaf samples of A. indica were collected. Bark pieces (5.0 × 5.0 cm) from the trunk were cut 1.5-2.0 m above the ground level with the help of sterile machete. The outer part was considered as bark and leaves were placed in polythene bags, labelled, transferred in ice box to the laboratory and placed in a refrigerator at 4°C. The samples were processed within 24 h of collection.

The samples were washed thoroughly in the running tap water before processing. Bark, inner bark, twig and leaf samples were surface sterilized by dipping in 70 % ethanol (v/v) for one min and 3.5% NaOCl (v/v) for 3 min, rinsed thrice with sterile water and dried. Bits of 1.0 × 1.0 cm size were excised with the help of a sterile blade. Three hundred segments representing bark, inner bark and leaf of A. indica were placed on water agar (15 g/ l) (WA) medium supplemented with streptomycin (100 mg/l; Sigma, St. Louis, MO, USA) contained in 9 cm diameter Petri dishes. Ten to fifteen segments were placed on solidified 20 ml WA medium in each Petri dish. The Petri

dishes were incubated at 22°C with 12 h light and dark cycles up to four weeks (Schulzet al., 1993). After sporulation, individual fungal colonies were picked from the edge with a sterile fine tipped needle and transferred onto potato dextrose agar (PDA) medium for further identification. The identification was done based on the morphological and conidial characteristics. All isolates were maintained in cryovials on PDA layered with 15% glycerol(v/v) at -80°C in an Ultrafreezer.

Isolation of PGPR

PGPR strains like P. fluorescens and B. subtilis were isolated from pepper growing regions and named as (strain A and B). The soil samples were serially diluted on nutrientmedium and PGPR strains were confirmed by biochemical characterizations.

Biochemical characterization

The gram reaction was determined following the staining procedure. First, thin bacterial smear was prepared on a clean glass slide, dried in air and fixed by heating. The dried smear wasflooded with crystal violet solution for 30 sec and washed in tap water for few seconds. It was again flooded with Grams iodine solution for 1 min and washed and blot dried. It was then decolourized with 95% ethyl alcohol by applying drop by drop until no more colour flows from smear, washed and blot dried. Finally, slides were counter stained for about 1 min with safranin, washed and examined under microscope using oil immersion objective.

KOH solubility test

A loopful of bacterial strains (strain A and B) from a well grown colony was mixed in a drop of 3% aqueous KOH solution for not more than 10 sec with the help of a toothpick. Toothpick was raised few centimeters from the glass slide and was observed for the formation of a mucoid thread.

Starch hydrolysis

The strains (A and B) were streaked on starch agar medium to evaluate their ability to hydrolyze starch (amylase production). The plates were incubated at 28°C and for 3-4 days. Starch hydrolysis was observed by flooding the plates with Gram's iodine solution for 30 sec. The appearance of clear zone around the growth of each bacteria indicated starch hydrolysis.

Kovac's test

A loopful of bacteria (strain A and B) was rubbed on a filer paper with drops of aqueous N,N,N,N-tera methyl-p-phenylenediamine dihydrochloride solution. Based on the standard procedure, isolates which developed purple colour within 10 sec were taken as positive, purple colour in 10-60 sec were taken as slow positive and those with no colour for more than 60 sec taken as negative to oxidase test.

Catalase test

A 24 h old culture of the bacteria (strain A and B) was flooded with 1 ml of 3% hydrogenperoxide and observed for the production of gas bubbles.

Lipase activity

Tween 80 agar medium was autoclaved and poured into sterile petri plates. The plates were streak inoculated with bacterial strains (strain A and B). The plates were incubated for threedays and

observed for the development of a milky precipitate around the colony. Cultures are positive for the lipase test, if granular precipitate is seen around the colonies.

Arginine dihydrolase test

A fresh culture tube containing 5 ml of sterilized Thornely's medium was stab inoculated with the bacterial strains (strain A and B). The surface of the medium was sealed with sterile molten Vaseline. Uninoculated tube served as negative control. The tubes were incubated at 26 ±2°C for three days and observed for the change in colour. Change in colour from orange to red indicates positive result.

Casein hydrolysis

Double strength nutrient agar medium with skimmed milk powder solution (10%) was sterilized in two different flasks. Both were mixed well before pouring into sterile petri plates. The bacterial strains (strain A and B) were streak inoculated with the test isolate and incubated at26 ± 2°C for 48 h. Bacterial strains were recorded positive if the zone of hydrolysis was seen around the colonies.

H_2S production from peptone

Test tubes containing reagent were autoclaved at 121°C for 20 min. Filter paper strips(0.5 × 7.5 cm) soaked in saturated solution of lead acetate were sterilized and dried in an oven at 60°C and placed at the mouth of the test tube in such a way that one half of the strip was hung below the cotton plug and the other half remained outside. Tubes were inoculated with bacterial strains (strain A and B). Uninoculated tube was maintained as negative control. Tubes were incubated at 26 ± 2°C for three days for the production of H_2S.

Levan formation

Nutrient agar medium supplemented with 5% sucrose and 0.2% (w/v) yeast extract was prepared, sterilized and poured into sterile petri plates. The medium was streak inoculated with the test bacterial strains (strain A and B) and incubated for 3-4 days. The plates were observedfor the development of white, domed, shining, mucoid colonies, which is due to the formation of levan by the enzyme levan sucrose produced by the bacteria.

Protease activity

Nutrient agar medium supplemented with Difco gelatin dissolved in distilled water was prepared and dispensed into sterile petri plates. Plates were inoculated with test bacterial strains (strain A and B) and incubated for two days at 26 ± 2°C after the incubation period, the plates were flooded with saturated ammonium sulphates solution to observe the zone of hydrolysis.

Cellulase activity

Cellulase medium was prepared and autoclaved. The plates were point inoculated withthe bacterial strains (strain A and B) and incubated for two days at 26 ± 2°C. After the incubation, the plates were flooded with 0.5% congo red for 15 min and then bleached with 1 M NaCl.

Gelatin liquefaction

The media were stab inoculated with bacterial strains (strain A and B) were grown for 48 h on yeast peptone sucrose agar medium and incubated at 26 ± 2°C After 3, 7 and 21 days of incubation, each isolate was evaluated for gelatin liquification. The bacterial strains in the test tubes were kept at 4°C for 30 min.

Litmus milk test

Litmus milk broth supplemented with skimmed milk was inoculated by PGPR strains Using sterile technique and appropriately labeled tube by means of a loop inoculation. The last tube will serve as a control. After inoculation of test cultures it was incubated for 24 to 48 hours at 37˚C.

Efficacy of rhizobacterial and endophytic fungal strains biocontrol agents on radial growth of P. capsici

Rhizobacterial strains P. fluorescens and B. subtilis and endophytic strains were T.asperellum and T. viride were tested against P. capsici by dual culture technique. The mycelial disc (9 mm) from seven days old culture of P. capsici was placed in one side of the petriplate containing 15 ml of PDA medium. After three days of pathogen inoculation, 24 h old bacterial strains P. fluorescens and B. subtilis strains were streaked on the opposite of the petriplate by the help of sterilized inoculation needle. Endophytic fungal strains T. asperellum and T. viride were streaked simultaneously against P. capsici. Three replications were maintained for each treatment. The plates were incubated at room temperature (23± 2°C) for three days and seven days, respectively, and inhibition zone was measured. The radial growth of the pathogen and per cent reduction over control was calculated by using the formula as follows;

$$\text{Per cent reduction over control} = \frac{\text{C-T}}{\text{C}} \times 100$$

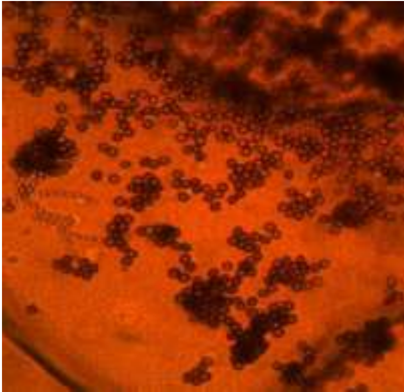
where, C – mycelial growth of the pathogen in control (mm) and T – mycelial growth of the pathogen in dual plate (mm).

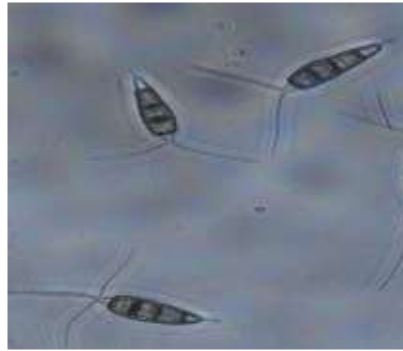
RESULTS

Identification of endophytic fungi

A total of 10 species with 28 isolates of endophytic fungi were recovered from 300 tissue segments of bark and leaf from A. indica recovered from 5 regions and 20 were identical in all the regions of plant tissues. Among the endophytes, Acremonium strictum was 1.33%. The other endophytes recovered from A. indica are Acremonium strictum, Fusarium oxysporum, Pestalotipsis sp., Aspergillus niger, Trichoderma viride, Trichoderma asperellum, Colletotrichum gloeosporioides, Fusarium solani, Aspergillus flavus and Phomopsis sp.

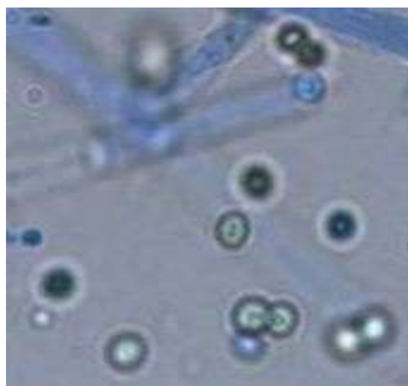
Acremonium strictum Fusarium oxysporum



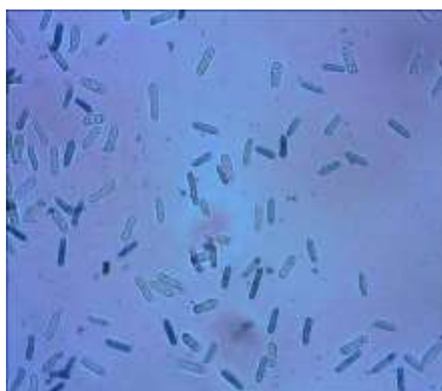


Aspergillus niger Pestalotiopsis sp.

Trichoderma viride



Trichoderma asperellum



Colletotrichum gloeosporioides

Fusarium solani

Aspergillus flavus

Phomopsis vexans

Grams staining

The bacterial strain A is indicated to be gram negative and strain B as positive and both appeared as rod shaped.

KOH solubility test

The bacterial strain A produced thin mucoid strand when a loopful of test bacteria was mixed with 3% KOH solution, indicating the positive for KOH solubility test and strain B does not produced any mucoid strand when mixed with KOH this indicates negative for KOH solubility test.

Starch hydrolysis

Strain A failed to produce a clear zone of hydrolysis when the culture plates were floodedwith Grams iodine and resulted in negative for starch hydrolysis. A clear zone of hydrolysis was formed around the strain B. Hence, the test was positive for starch hydrolysis.

Kovac's oxidase test

The colour of the strain A did not turn even after 10 sec of rubbing on the filter paper impregnated with Kovac's oxidase reagent and resulted in negative for Kovac's. Strain B turn blue after 30 sec of rubbing on filter paper impregnated with Kovac's oxidase reagent and resulted in positive.

Catalase test

Bubbles were not formed when the strain A culture were flooded with 3% H_2O_2 indicating the strain A as catalase negative. Bubbles were formed when the strain B were floodedwith H_2O_2 indicating the strain B is positive for catalase.

Lipase activity

The formation of white precipitate around the colonies of strain A was absent. Hence, thebacterium was negative. Strain B formed white precipitate around the colonies and lipase was present in Strain B and hence the result is positive.

Arginine dihydrolase test

Change in colour from orange to red was not observed in tubes inoculated with bacterial strain A indicating negative for Arginine dihydrolase reaction. Tubes inoculated with strain B showed the change in the colour of the medium from orange to red indicating positive reaction.

Casein hydrolysis

Zone of hydrolysis was not observed around the colonies of the strain A, hence, the test isnegative for casein hydrolysis. Strain B showed clear zone of hydrolysis around the colonies indicating positive reaction

H_2S production

Filter paper strips impregnated with lead acetate turned black in tubes inoculated with thetest isolates due to the production of H_2S, which turns lead acetate to lead sulphide. No colour change was observed in strain B.

Levan formation

White domed, shiny mucoid colonies were observed when the medium was inoculated with strain A and hence the result is positive for Levan and Strain B did not showed any white, domed, shiny mucoid and the result indicates negative.

Protease activity

Clear zone of hydrolysis was not observed around the colonies of strain A when culture plates were flooded with saturated ammonium sulphate solutions, which indicates negativereaction. Zone of hydrolysis was observed around the colonies of strain B.

Cellulase activity

Clear zone of hydrolysis was not seen around the colonies of strain A when the culture plates were flooded with 0.5 Congo red and then bleached with 1M NaCl, hence, the test is negative for strain A. Zone of hydrolysis was observed around the colonies in strain B and the result indicates positive.

Litmus milk test

Clot was formed after the incubation time and results in negative for both the cultures.

Gelatin hydrolysis

Liquification of the medium was observed in the strain A tube indicated the positive for gelatin hydrolysis and no change in the medium was observed in strain B which showed negativeresult. Results are tabulated in table 1.

Table 1: Biochemical characterization of PGPR strains

Sl. No.	**Biochemical test**	**P. fluorescens (Strain A)**	**B. subtilis (Strain B)**
1	Grams test	**Negative**	Positive
2	KOH solubility test	+	-
3	Kovac's hydrolysis	+	+
4	Catalase test	+	+
5	Casein hydrolysis	**-**	+
6	Action on litmus milk	**-**	-

7	Lipase activity	-	+
8	Arginine dihydrolase	-	+
9	H_2S production	+	+
10	Levan formation	+	+
11	Protease activity	-	+
12	Cellulase activity	-	+
13	Starch hydrolysis	-	+
14	Gelatin hydrolysis	+	-

+: positive and -: negative

Effect of biocontrol agents on radial growth of P. capsici

PGPR strain of B. subtilis, P. fluorescens and endophytic fungal strains of T. viride and T. asperellum were tested individually to assess the radial growth of P. capsici. All the treatments were effective in reducing the mycelial growth of the pathogen. However, B. subtilis had resulted with mycelial growth of 50 mm and inhibition zone of 25 mm and P. fluorescens showed 63 mm of mycelial growth and 18 inhibition zone, respectively. Trichoderma viride recorded the maximum inhibition zone of 26 mm, and T. asperellum showed 19 mm inhibition zone and 50mm and 60 mm mycelia growth, respectively. The control plates recorded the highest mycelialgrowth of 91 mm (Table 2).

Table 2: Effect of biocontrol agents on the mycelial growth of P.capsici

Sl. No	Treatments	Mycelial growth (mm)	Inhibition zone (mm)
1	Bacillus subtilis	50±0.32	25±0.50
2	Psuedomonas fluorescens	63±0.22	18±0.28
3	Trichoderma asperellum	59±0.5	19±0.50
4	Trichoderma viride	49±0.32	26±0.50
5	**RIDOMIL** GOLD	56±0.32	17±0.50
6	Control	91±0.12	0.00

Values are mean of three replications. In a column, mean followed by a common letter (s)are not significantly different at the 5% level by DMRT

Discussion

Endophytic microorganisms are a significant reservoir of novel bioactive secondary metabolites. The number of secondary metabolites produced by fungal endophytes is larger than that of any other endophytic microorganism class. These endophytic fungi are obviously a rich and reliable source of chemically novel bioactive compounds with huge medicinal and agricultural potential (Strobel et al., 2004). The Western Ghats is one of the hot spots of plant biodiversity endowed with more than 4,700 different plant species. A new species of endophyte, Muscodor kashayum was recently isolated from the medicinal species Aegle marmelos (Bael tree) from the Western Ghats of Muthanga region of Wayanad Wildlife Sanctuary, Kerala, India. Azadirachta indica is an important medicinal plant used in the traditional medicine and has a variety of pharmacological activities.

The findings proved that the number of endophytic fungi was higher in bark than in leaves. However, the overall colonization frequencies differed with different organs. The number of species occurring in the inner bark and the twig region was almost the same; yet, the twig was more densely colonized by the endophytes as evidenced by the total CF%. Similar results have been reported for Azadirachta indica (Rajakumar et al., 2012). However, the colonization

frequency reported according to Meng and Chen (2001) was 62.5% where a total of 32 species belonging to 21 genera were isolated from inner bark of Prosopis cineraria tree. Among the plant parts, the colonization as well as the isolation rates of endophytic fungi was higher in bark followed leaf samples. Study corroborates with the findings of Sun et al. (2008), who have reported that colonization frequency and isolation rates of endophytic fungi were conspicuously higher in bark than in leaves in the plants examined.

Two strains of rhizobacterial isolates and two fungal endophytes were used for theefficacy study of foot rot disease. These isolates also induced the systemic resistance against P. capsici and in turn they enhanced the plant height. The mode of pathogen infection that excludes direct penetration in host cells, suggests the possibility that products of chitinase activity posses some antimicrobial activity. The present study demonstrated that the isolates of rhizobacterial B. subtilis, endophytic fungi T. viride and T. asperellum consistently reduced the radial mycelial growth of P. capsici by producing various antibiotics and reduced the foot rot of pepper under green house and field conditions by inducing ISR compared to individual agents.

Conclusion

The present study, demonstrates that PGPE and PGPR are due to isolates of T. viride and

B. subtilis and their role in enhancing growth of vines against the foot rot disease of pepper. Moreover, it is concluded that T. viride and B. subtilis increase the plant growth and resistance to P. capsici. It is easily imagined that the different isolates of rhizobacterial strains and endophytic fungal strains produces antimicrobial products and defense enzymes restrict the development of challenging phytopathogenic fungi.

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