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Pseudomonas fluorescens (FP7) amended with chitin bioformulation for the management of anthracnose pathogen in mango cultivar Alphonso

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Abstract: **Certain Plant Growth-Promoting Rhizobacteria (PGPR) and yeast antagonists were evaluated against mango anthracnose incited by *Colletotorichum gloeosporioides* (Penz.) both *in vitro* and *in vivo* conditions. Among the antagonists, *Pseudomonas fluorescens* (FP7) significantly reduced the in vitro growth of *C. gloeosporioides*. For field evaluation, commercial cultivar Alphonso was selected. Talc-based bioformulations with and without chitin amendment were prepared and sprayed at 15 days and 30 days intervals. The spray was initiated from pre-flowering stage with a concentration of 0.5%. Among the bioformulations, *P. fluorescens* (FP7) amended with chitin sprayed at 15 days intervals was found to be significant in reducing the anthracnose incidence and subsequently increased panicle number, mean number of fruits and fruit yield.**

Key words: Mango, Anthracnose, Pseudomonas fluorescens, Saccharomyces cerevisiae, Bacillus subtils, Chitin

Introduction

Mango (*Mangifera indica* L.) cultivation is an important agribusiness in India. Even though it has the largest area, the productivity is very low due to various biotic and abiotic strains. Of them, anthracnose disease incited by *Colletorichum gloeosporioides* (Penz.) is a highly destructive pathogen that causes yield loss upto 60% or more during heavy rainy season (Arauz, 2000). The application of chemical fungicide is still the major method of controlling this disease. The frequency of fungicide application per season has ranged from one to sixteen sprays depending on the mango grower. In recent years, occasional reports from growers have indicated that the control efficacy of the fungicide seems to have dwindled and concerns regarding resistance of the pathogen to the fungicide have been increasing. Hence, the present study was undertaken to test the various biocontrol agents as alternative method to suppress the anthracnose pathogen in field conditions.

Materials and methods

Isolation of pathogen and proving the pathogenicity

The pathogen was isolated from the anthracnose infected fruit samples collected from different agro climatic zones comprising major mango growing areas and markets of Tamil Nadu. A small bit of peel samples containing both infected and healthy portions were surface sterilized with 1 per cent sodium hypochlorite solution for 1 min. and subsequently washed three times with sterile water. Then they were transferred into a sterile Petri dish containing potato dextrose agar (PDA) medium amended with streptomycin. The plates were then incubated at room temperature (28±2°C) for the growth of fungal culture.

For proving the pathogenicity, the spore suspension of C. *gloeosporioides* isolates containing 10^5 spores/ ml were inoculated into fruits showing uniform size, free from bruise or blemish at full-three quarter stage of maturity by pin prick method (Swinburne, 1976). Commercial cultivar Alphonso was selected. Inoculation was done at two equidistant points and placed in perforated polythene bags to maintain high humidity and incubated at room temperature (28±2°C). The symptom expression was recorded five days after inoculation.

Collection of biocontrol microorganisms

The biocontrol strains of *Pseudomonas fluorescens* (Pf1), *P. fluorescens* (FP7), *Bacillus subtilis* (Bs-1) and *Saccharomyces cerevisiae* (Sc-1) were collected from Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India. The bacterial strains were identified by following the Bergey's Manual of Systematic Bacteriology (Hildebrand *et al.* 1992) and for yeast according to Kreger-van Riji, (1984).

Efficacy of biocontrol strains under in vitro conditions

For *in vitro* screening of biocontrol agents against C. *gloeosporioides*, the microbes were streaked on one side of a Petri dish (1 cm from the edge of the plate) with PDA medium and a mycelial disc (8 mm diameter) of seven day old culture of *C. gloeosporioides* isolate was placed on the opposite side of the Petri dish perpendicular to the bacterial streak (Nandakumar *et al.* 2001). The plates were incubated at room temperature (28±2°C) for four days and the zone of inhibition was measured.

Preparation of biocontrol formulations

For the development of bioformulation of biocontrol agents, a talc based formulation was developed. The PGPR on King's B broth (*Pseudomonas fluorescens*), Nutriennt Agar (*Bacillus subtilis*) and yeast strains on Yeast Extract Glucose Agar (*Saccharomyces cerevisiae*) were grown with constant shaking at 150 rpm for 48 h at room temperature (28±2°C). The suspension cells were harvested and centrifuged at 6000 rpm for 15 min. and resuspended in phosphate buffer (0.01 M, pH 7.0). The concentration of cells were adjusted using a spectrophotometer to approximately 10^8 cfu/ ml (OD_{595}=0.3) and used as suspension inoculum (Thompson, 1996).

Incorporation of colloidal chitin and formulation development

Five gram of crab shell chitin (Sigma, USA) was slowly added into 100 ml of 0.25 N HCl with vigorous stirring and kept overnight 4°C. The mixture was filtered through glasswool into 200 ml of ice cold ethanol at 4°C with rapid stirring. The resultant chitin suspension was centrifuged at 10000 rpm for 20 min and the chitin pellets were washed repeatedly with distilled water until the pH became neutral. The concentration of colloidal chitin was adjusted to 10 mg per ml.

Colloidal chitin was incorporated into broth medium (1% v/v) and the mixture was autoclaved at 150 psi for 30min. Then the cultures were inoculated individually into their respective broth and kept in shaker for 48 h at room temperature (28±2°C). After 48 h of incubation, the broth containing 9 x 10^8 cfu/ ml was used for the preparation of talc-based formulation. To 400 ml of microbe suspension, 1 kg of the purified talc powder (sterilized at 105°C for 12 h), calcium carbonate 15 g (to adjust the pH to neutral) and carboxymethyl cellulose 10 g (adhesive) were mixed under sterile conditions. The product was shade dried to reduce the moisture content (less than 20 per cent) and then packed in polypropylene bag and sealed. At the time of application, the population of biocontrol strains in talc formulation was found to be 2.5 to 3 x 10^8 cfu/g.

Efficacy of preharvest application of biocontrol formulations under endemic field conditions

For studying the efficacy of biocontrol formulations on disease development and its relation with yield parameters, a field trial was conducted at Bodinaikkanur (South Tamil Nadu), where the anthracnose disease is endemic. In the selected fields, the anthranose incidence ranged from 70 to 80 per cent (cv. Alphonso) in the previous year. Talc-based bioformulation was prepared and sprayed @ 5g/ litre. The

Table 1. Antagonistic effect of biocontrol strains against *C. gloeosporioides* under *in vitro* conditions

Biocontrol stains	Radial growth (cm)*	Per cent inhibition over control
P. fluorescens (Pf1)	5.05^{c}	37.9
P. fluorescens (FP7)	3.30^{a}	59.0
B. subtilis (Bs-1)	4.70^{c}	42.2
S. cerevisiae (Sc-1)	3.88^{b}	52.3
Control	8.13^{d}	

* Values are means of four replications. In a column, means followed by common letter are not significantly different at 5% level by DMRT.

Table 2. Efficacy of preharvest application of bioformulations on flower induction and anthracnose incidence in mango cv. Alphonso

Treatments	Spray intervals	No.of Panicles/ m^2	Fruit infection (per cent)
P. fluorescens (Pf1)	15 days	25^{cde}	55.7^{de} (54.16)*
P. fluorescens (FP7)		26^{cd}	40.3^{k} (39.4)
B. subtilis		23^{efg}	61.5^{fg} (51.64)
S. cerevisiae		22^{fgh}	29.0^{l} (32.56)
Pf1 + chitin		24^{def}	28.5^{l} (32.25)
FP7 + chitin		30^{a}	12.6^{n} (20.7)
B. subtilis + chitin		29^{ab}	45.4^{ij} (42.36)
S.cerevisiae + chitin		25^{cde}	57.5^{h} (49.32)
Carbendazim		20^{hi}	33.3^{l} (33.4)
Chitin		20^{hi}	69.2^{cd} (56.31)
P. fluorescens (Pf1)	30 days	18^{j}	7.0^{o} (56.81)
P. fluorescens (FP7)		25^{cde}	46.9^{i} (43.22)
B. subtilis		21^{gh}	75.0^{b} (60.0)
S. cerevisiae		18^{j}	40.7^{k} (39.63)
Pf1 + chitin		23^{efg}	44.7^{ij} (41.95)
FP7 + chitin		27^{bc}	24.2^{m} (29.44)
B. subtilis + chitin		26.5^{c}	59.4^{gh} (50.43)
S.cerevisiae + chitin		23^{efg}	64.1^{ef} (53.2)
Carbendazim		18^{j}	42^{jk} (40.39)
Chitin		18^{j}	76.0^{b} (60.70)
Control		15^{j}	83.3^{a} (65.94)

Values are means of three replications with each replicate consisted of five trees. In a column, means followed by a common letter are not significantly different at 5% level by DMRT.

* Values in parenthesis are arc sine transformed values.

spray was given from pre flowering stage to fruit maturity stage. The experiments were laid out with randomized block design and three replications were maintained in each treatment and each replicate consisted of five trees. The treatments were *P. fluorescens* (Pfl), *P. fluorescens* (FP7), *B. subtilis*, *S. cerevisiae*, each with and without chitin amendmennnnt, carbendazim (0.1%), chitin (1%) and control (water spray). The biocontrol formulations were applied at 15 days and 30 days interval. In both the trials, about 15 years old trees of cultivar, Alphonso with uniform growth susceptible to anthracnose maintained under uniform cultural operations were sprayed along with suitable control at the time of pre-flowering stage in second week of November 2001.

Efficacy of preharvest application of biocontrol formulations on anthracnose incidence, panicle initiation and yield attributes

For studying the efficacy of preharvest application of biocontrol formulations on panicle initiation and disease development, observations on mean number of panicles/m^2 mango tree foliage area (Rangaswamy, 1995) at the time of flowering and the percentage of anthracnose affected fruits at pre-harvesting stage were recorded. The yield parameters of the sprayed trees were evaluated at harvesting stage by determining (i) the mean number of fruits (ii) mean fruit weight and (iii) fruit yield. All the parameters were recorded in each replication and extrapolated to per hectare.

Results

Isolation of pathogen and proving the pathogenicity

The anthracnose disease affected mango trees (cv. Alphonso) were selected and the isolation of the fungi associated with the disease was made. The fungus was isolated in pure culture from the infected mango fruits. Based on the morphology and cultural characters, culture was identified as *Colletotrichum gloeosporioides* according to Ekbote *et al.* (1997). This fungal culture was initially pink that produced conidia with oil globules which later turned out to whitish grey and subsequently became black. The length and width of conidia was measured to be 8.3 to 27.4 µm and 2.0 to 6.6 µm respectively.

The mango fruits inoculated with *C.gloeosporioides* produced typical symptoms of anthracnose disease within five days after inoculation. Initial symptoms *viz.* brown to black lesions with an indefinite border appeared on the fruit surface within four days after inoculation. They coalesce and covered the extensive areas of fruit as tear-strain pattern developing from the basal toward the distal end of the fruit. Lesions spread from peel to pulp on day seven after inoculation leading to entire fruit decay.

Efficacy of biocontrol strains on growth of Colletotrichum gloeosporioides.

Among the biocontrol strains evaluated for their efficacy in suppressing the mycelial growth, the rhizobacteria *P. fluorescens* FP7 revealed higher inhibition zone formation than other isolates tested and control. The strain FP7 exhibited the maximum pathogen suppression (59 per cent) followed by S. *cerevisiae* (52.3 per cent) (Table 1).

Efficacy of preharvest application of biocontrol formulations on panicle initiation

While evaluating the biocontrol formulations in field trials, number of panicles/ m^2 was calculated after third spray by random sampling method (three replications with 50 samples/ replication). The results indicate that flower initiation in PGPR and yeast treated plants were greater than untreated control. The trees sprayed with FP7 + chitin bioformulation at 15 days intervals recorded maximum panicle number (30/m^2) followed by *B. subtilis* + chitin (29/m^2). The minimum number of panicles was recorded by unsprayed control (15/m^2) (Table 2).

Table 3. Efficacy of preharvest application of biocontrol formulations on yield attributes in mango cv. Alphonso

Treatments	Spray intervals	Mean no.of fruits/ha	Fruit weight (kg/fruit)	Fruit yield (t/ha)
P. fluorescens (Pf1)	15 days	38000^{ijk}	0.253^{e}	9.61^{ij}
P. fluorescens (FP7)		52000^{de}	0.256^{de}	13.31^{e}
B. subtilis		39000^{ij}	0.26^{de}	10.14^{hi}
S. cerevisiae		62000^{b}	0290^{a}	16.74^{j}
Pf1 + chitin		54000^{d}	0.258^{de}	13.93^{de}
FP7 + chitin		79000^{a}	0.270^{bc}	22.91^{a}
B. subtilis + chitin		44000^{h}	0.264^{cd}	11.62^{f}
S. cerevisiae + chitin		40000^{i}	0.265^{cd}	10.60^{gh}
Carbendazim		57000^{c}	0.277^{b}	15.79^{c}
Chitin		39000^{ij}	0.260^{de}	10.14^{hi}
P. fluorescens (Pf1)	30 days	37000^{ijk}	0.196^{ij}	7.14^{k}
P. fluorescens (FP7)		49000^{fg}	0.200^{ij}	9.60^{ij}
B. subtilis		36000^{k}	0.200^{ij}	7.20^{k}
S. cerevisiae		54000^{d}	0.230^{f}	11.34^{fg}
Pf1 + chitin		47000^{g}	0.197^{ij}	9.26^{ij}
FP7 + chitin		62000^{b}	0.210^{gh}	14.26^{d}
B. subtilis + chitin		37000^{ijk}	0.203^{hi}	7.51^{k}
S. cerevisiae + chitin		39000^{ij}	0.201^{hij}	7.84^{k}
Carbendazim		50000^{ef}	0.213^{g}	10.65^{gh}
Chitin		38000^{ijk}	0.202^{hij}	7.68^{k}
Control		26000^{l}	0.163^{j}	5.02^{l}

Values are means of three replications with each replicate consisted of five trees. In a column, means followed by a common letter are not significantly different at 5% level by DMRT.

Efficacy of preharvest application of biocontrol formulations on anthracnose incidence

The anthracnose disease incidence was assessed at fruit maturity stage. The mean percentage of infected fruits in bioformulation treatments ranged between 12.6 to 70 per cent compared to untreated control (83.3 per cent). The rhizobacteria FP7 + chitin recorded significantly very low anthracnose in fruits (12.6 per cent) with 84 per cent reduction over control (Table 2).

Efficacy of preharvest application of biocontrol formulations on yield attributes of mango

While evaluating the yield attributes of mango, FP7 + chitin treated Alphonso trees recorded significantly high mean number of fruits (79000/ ha) and fruit yield (22.9 t/ ha) than fruit number (26000/ ha) and yield (5.02 t/ ha) in untreated control. The per cent increase over control was 356% (mean number of fruits/ ha) and 203% (mean yield/ ha). The maximum fruit weight was recorded in *S. cerevisiae* treatment

(0.29 kg/ fruit) compared to untreated control (0.163kg/ fruit). The bioformulations sprayed at 15 days intervals recorded maximum yield attributes when compared to 30 days spray intervals (Table 3).

Discussion

In recent years, anthracnose has become a major challenge to both the pathologists working with the pathogen and to mango researchers in general. The routine control measure involving chemical pesticide application leads to toxicity, residual effect and resistance development by pathogens (Koomen and Jeffries., 1993). The current situation is mainly focused on biological control. Since all the commercial mango varieties are susceptible to the disease, biological control provides an effective, persistent and durable protection. The plant growth- promoting rhizobacteria (PGPR) and yeasts are reported for the control of many fungal pathogens (Nandakumar *et al.* 2001). This concept has exploited the potential of the PGPR and yeast strains into commercialization of a number of microbial biocontrol products. Of which, talc-based bioformulations have been reported in the management of several crop diseases (Ramamoorthy *et al.* 2001).

The present investigation clearly indicated that the PGPR strain FP7 amended with chitin bioformulation significantly suppressed anthracnose incidence under endemic conditions. The amendment of chitin enhanced its activity in the field thereby substantial increment in yield attributes and reduced fruit infection was recorded. This finding is in agreement with previous observations by Radja Commare *et al.* (2002), who tested the efficacy of *P. fluorescens* (Pfl) and *P. fluorescens* (FP7) talc-based formulations with and without chitin amendment in rice against sheath blight infection. The foliar application of Pfl with chitin, FP7 with chitin recorded lower per cent disease index (PDI) of 22 and 23 respectively, compared to the untreated control (75 PDI) in field conditions. Both tested formulations resulted in enhanced yield of 6.3 t/ha while a lower yield of 5.8t/ha was recorded in the untreated control. Bharathi (2001) reported that the PGPR strains *viz.* Pfl and *B. subtilis* amended with chitin, significantly reduced the *Colletotrichum capsici* incidence in chilies. Saravanakumar (2002) reported that PGPR strain Pfl amended with chitin significantly reduced the dry root rot of greengram in field conditions and the maximum disease reduction of 82.13 per cent over control was recorded. The highest yield of 1218 kg/ ha was recorded by Pfl + chitin which was significantly different from untreated control plots (583 kg/ ha). Similar enhanced biocontrol efficacy by fluorescent pseudomonads against *Colletotrichum falcatum* in sugarcane was reported by Viswanathan and Samiyappan (2001), for which the reason attributed was increased multiplication of the PGPR and enhanced chitinase activity.

Since it is well known that anthracnose symptoms and its consequential effects on yields are most severe when trees are infected in early growth stages, the pre- flowering application in this investigation can reduce the initial infection. The study revealed that the bioformulations drastically reduced anthracnose in field conditions when they were applied at pre -flowering stage. Anthracnose infected trees treated with FP7 and chitin showed maximum recovery of 87 per cent fruits and the yield or quality was not affected compared to the untreated control. The application of FP7 with chitin at fortnightly intervals recorded significant increase in fruit yield compared to monthly interval spray, other biocontrol formulations and the unsprayed control. Similar trends were noticed on strawberry, where the field application of biocontrol agents at the flowering stage was found to be effective in suppressing the incidence of *Botrytis cinerea* on stamens, petals and fruit (Peng *et al.* 1992). The yeast -like fungus *Auriobasidium pullulans* and the yeast *Candida oleophila* were more effective against *Botrytis cinerea* (storage rots on strawberry) when applied at blooming stage

compared to applications immediately after harvesting (Lima *et al.* 1997).

Kloepper *et al.* (1980) have indicated that PGPR application was often associated with increased rates of plant growth. PGPR and yeast antagonists promote plant growth by synthesizing phytohormones like gibberellins, cytokinins and indole acetic acid (Ramamoorthy and Samiyappan, 2001). In the present investigation, an enormous quantity of panicle formation was observed in specific treatments *viz.* FP7 + chitin followed by *B. subtilis* + chitin.

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