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Induction of systemic resistance in chilli (*Capsicum annuum* L.) by *Pseudomonas aeruginosa* against anthracnose pathogen *Colletotrichum capsici*

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Abstract

Colletotrichum capsici Butler and Bisby, the causal agent of chilli anthracnose disease, sternly affects chilli yield and quality worldwide. Biocontrol agents and resistance inducers could be a valid alternative to chemical pesticides. The capability of two phosphate solubilizing *Pseudomonas aeruginosa* isolates *viz*., Ps 2 and Ps 3 (KR270346 and KR270347) against chilli anthracnose both under *in vitro* and *in vivo* conditions was demonstrated. *Pseudomonas aeruginosa* Ps 2 showed maximum mycelial growth inhibition of 93.41% and the isolate Ps 3 showed 72.5% inhibition of *Colletotrichum capsici* in dual culture inhibition assay after 5 days of incubation. There was a greater reduction of anthracnose infection caused by *C. capsici* on matured fruits in *Pseudomonas aeruginosa* treated plants when compared to untreated control. The strain possessed chitinolytic and proteolytic activities, produced HCN, siderophores, and was able to produce salicylic acid at a moderate level. The isolate produced amylase. However both of them are unable to produce lipase. The isolates induced systemic resistance in chilli corroborated with increased levels of phenylalanine ammonia lyase, peroxidase and polyphenol oxidase under greenhouse experiments. The results indicate the potential of the indigenous *Pseudomonas aeruginosa* isolates as biocontrol agents against chilli anthracnose.

Keywords: Bacterial antagonists, Biocontrol, Chilli, *Colletotrichum capsici,* Dual culture assay**,** Siderophores.

Introduction

Chilli (*Capsicum annuum* L.) is an important vegetable crop due to its large scale consumption as a seasoning vegetable in India and many other countries as well. The crop suffers from many diseases like damping off, foot rot, anthracnose, dieback, fruit rot, wilt, leaf spots, powdery mildew etc. Anthracnose caused by *Colletotrichum* sp. is a major problem in India and results in both pre- and post-harvest fruit decay with yield losses of up to 50% (Pakdeevaraporn et al., 2005). The different species of *Colletotrichum* causing chilli anthracnose reported from India include *Colletotrichum acutatum, C. capsici, C. coccodes, C. dematium, C. gloeosporioides* and *C. siamense* (Saini et al., 2016). Economic losses caused by the disease are mainly attributed to lower fruit quality. The fungus very commonly occurs in chilli growing areas of India resulting in disease incidence levels ranging between 66% and 84%, and causing yield loss up to 12-50% (Bagri et al., 2004; Sharma et al., 2005). Typical anthracnose on chilli fruit include sunken necrotic tissues, with concentric rings of acervuli and fruits showing blemishes have reduced marketability (Manandhar et al., 1995). Many species of *Colletotrichum* are seed borne and they may survive in soil on debris and may be spread by water splash dispersal of conidia and transmission

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of ascospores through the air (Nicholson and Moraes, 1980). They are capable of growing in and on seeds as acervuli and micro sclerotia (Pernezny et al., 2003). Chemicals are mainly used in the control of chilli anthracnose and the fungicide traditionally recommended for anthracnose management in chilli is manganese ethyl-enebisdithiocarbamate (Maneb) (Smith, 2000), although it does not consistently control the severe form of anthracnose on chilli fruit. Continuous use of these chemicals led to new challenges like development of pest resistance, food poisoning, environmental pollution, negative effect on farmers health, and increase in cost. To overcome the undesirable effects of chemical usage, there is a need to incorporate alternative control components that are effective in the field.

The diversity of naturally occurring microorganisms of the rhizosphere and phyllosphere and their potential for biological control of plant pathogens have been examined extensively (Jayraj et al., 2007). Pseudomonads are considered to be important rhizosphere microorganisms, and considerable research is underway globally to exploit their potential. A number of fluorescent pseudomonads have been reported to have *in vitro* and *in vivo* biocontrol potential against a wide range of phytopathogens (Gupta et al., 2002; Kishore et al., 2005; Mansoor et al., 2007).

The inhibition of growth of pathogenic microorganisms is mostly due to the ability of bacterial antagonists to synthesize antifungal compounds, antibiotics, cyanide or siderophores. *P. aeruginosa* strain produces numerous compounds which are responsible for disease control. These inhibitory compounds are siderophores, HCN, degradative extracellular enzymes such as chitinase, protease, cellulose, â -1, 3 glucanase and antibiotics such as pyrrolnitrin, pyoluteorin, phenazine (Haas and Defago, 2005; Deshwal et al., 2011) and activation of plant disease resistance. Selected strains of non-pathogenic microorganisms are able to reduce plant disease through activation of a plant-mediated defense mechanism known as induced systemic resistance (ISR) (Van Loon, 2007).

ISR has been shown to provide protection against different types of pathogens in several plant species (Pieterse et al., 2002). After leaf and root treatment with ISR-inducers, the resistance is systemically activated in untreated tissues, extending to aboveground plant parts, a disease protection phenotypically similar to pathogen-induced systemic acquired resistance (SAR). Biological control using introduced microorganisms with the capacity to elicit ISR against plant diseases has been extensively studied under greenhouse and field conditions (Akram et al., 2015). Thus, in recent years, the induction of systemic resistance has been studied as a new potential measure for controlling crop diseases in the field, and a number of microorganisms and chemical inducers have been examined in various plant–pathogen systems. The present study was proposed with an objective to evaluate the *in vitro* and *in vivo* antagonistic activity of *Pseudomonas aeruginosa* isolates PS2 and PS3 against a phytopathogenic fungi *C. capsici* and to elucidate the mechanism of antifungal activity.

Materials and methods

Materials

All the culture media were purchased from Hi Media, Mumbai, India. Colloidal chitin was obtained from Sigma Chemical Co., Bangalore. All other chemicals used were of analytical grade.

Organisms

The pathogenic organism was isolated from the infected fruits of *C. annuum* (Fig. 1) as a pure culture on potato dextrose agar (PDA) medium. The fruit specimens were washed with tap water, the discoloured parts cut into small pieces (5 mm), sterilized with 0.1% sodium hypochlorite (NaOCl) for two min., rinsed three time in sterile water, and dried between folds of sterilized filter paper. The sterilized fruit pieces were transferred to sterilized oat meal agar plates and incubated at room

temperature for 5 days. Mycelial bits were transferred to sterile petridishes containing oatmeal agar medium; later it was purified by the hyphal tip method and transferred to potato dextrose agar (PDA) slants and pure cultures of the pathogens were maintained for further studies.

Pseudomonas aeruginosa isolates were isolated from the samples of chilli (*Capsicum annuum* L.), fields around India by enrichment culture technique. The isolates were morphologically and biochemically characterized and identified using 16S rDNA sequencing technique and phylogenetic analysis (Linu et al., 2009; Linu et al., 2017). Their sequences were deposited in Genbank with accession numbers KR270346 and KR270347 .The cultures were maintained on Pikovskaya's agar slants at 6 °C in a refrigerator with regular subculturing (Pikovaskaya, 1948).

Dual culture assay

To determine the potential antifungal activity of the selected bacterial isolate *in vitro,* isolates were co-cultured alongside the pathogenic fungus *Colletotrichum capsici*. The fungal strains were cultured on PDA plates for 5 days at 28 °C; bacterial strains were grown in LB liquid medium for 24 h at 37 °C (Barhate et al., 2012). Competitive interactions between the bacterial isolates and *Colletotrichum capsici* were evaluated in dual-culture experiments in 90-mm Petri dishes containing 20 ml of PDA. Mycelial discs (5 mm in diameter) from fungal colonies and 2 ìl of bacterial suspension from the LB cultures were placed on the agar surface, 30 mm apart. Control cultures were inoculated with 2 ìl of distilled water instead of bacteria. Immediately after inoculation, the plates were sealed with plastic film and incubated at 28 °C in the dark for 3–5 days. Visual observations on the inhibition of growth of fungal pathogen were recorded after 5 days of incubation in comparison with the PDA plate simultaneously inoculated with only the fungal pathogen. The percentage reduction in growth was calculated following the formula

$$\text{Percentage of inhibition (\%)} = \frac{C - T}{C} \times 100$$

Where, C-diameter of fungal growth in control plate
T-diameter of fungal growth in test plate

In vitro antifungal bioassay

Production of volatile substance (HCN) and its effect on growth of C. capsici

The screening of the isolates for the production of hydrogen cyanide was done using the method described by Shabana mol et al. (2017). Here, filter paper strips soaked in .0.5% picric acid reagent were placed in the lid of petri dish and the lids were closed. The plates were sealed with parafilm and incubated for 30±10ºC for 48 hrs. The colour change of the filter paper pad was noted by a method described by Lee et al. (2008). The inhibitory effect of HCN on the growth of *C.capsici* was determined by the 'inverted plate technique' where, the pathogens was grown in PDA. The reduction in growth of fungal pathogen was calculated using the formula (Kazempour, 2004):

$$\text{Reduction in growth of fungal pathogen} = \frac{\text{Growth in control} - \text{Growth in antagonist inoculated}}{\text{Growth in control}}$$

Non volatile organic compound production

In order to study the effect of nonvolatile compounds, bacterial antagonists were grown on King's B broth and the pathogen was grown on PDA plates. The culture filtrate after 7 days of incubation was filtered through Millipore filters using vacuum pump assembly. 5ml of the culture filtrate was mixed with 20 ml PDA just before pouring into petri dishes and after cooling, inoculated with 5 mm discs of the pathogen. Pathogen inoculated without culture filtrate served as control. The colony diameters of the pathogen after 7 days were recorded and compared with the control.

Chitinase bioassay

The isolates were inoculated on LB plates supplemented with 0.5% colloidal chitin. Plates were incubated at 30ºC for 3 days. Enzymatic

activity was detected by the development of clear halo zones around the bacterial spots (Basha and Ulaganathan, 2002)

Detection of ammonia

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48–72h at 36±2°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production (Cappuccino and Sherman, 1992).

Detection of hydrolytic enzyme activity

Production of hydrolytic enzyme was qualitatively assayed in minimal medium containing gelatin, starch, pectin and carboxy- methyl cellulose (CMC) for protease, amylase, pectinase and cellulase. Plates were incubated for 48 h at 30°C and the formation of a clear zone around bacterial colonies was interpreted as being positive (Kasana et al., 2008).

Siderophore production.

Chrome Azurol S (CAS) agar medium was prepared as described by Schwyn and Neilands (1987) to detect siderophore production. CAS agar (blue agar) was inoculated at the center of the plate with 24-h-old culture of isolates and kept for incubation at 30°C for 72 h. The change of blue colour of the medium to orange or the presence of a yellow to light orange halo zones surrounding the bacterial colony indicated the production of siderophores.

Production of salicylic acid (SA)

To determine salicylic acid production of the isolates, the strains were grown in the standard succinate medium (SSM) at 28±2ºC for 48 hrs on a shaker incubator at 180 rpm. Cells were harvested by centrifugation at 10000 rpm for 5min and were re suspended in 1ml of 0.1 M phosphate buffer. 4 ml of the cell free culture filtrate was acidified with 1N HCL to pH 2.0 and SA were extracted with equal volume of chloroform. Four ml of water and 5 ml of 2M ferric chloride were added to the pooled chloroform phases. The absorbance of the purple iron-SA complex, which was developed in the aqueous phase, was read at 527 nm against blank in UV- visible spectrophotometer. A standard curve was prepared with SA in SSM and quantity of SA produced was expressed as mg/50 ml.

ACC deaminase production

The isolates were screened for ACC deaminase activity using the methods described by Dworkin and Foster (1958). For this, the isolates were inoculated on to DF minimal salt medium amended with 2 g/L ammonium sulphate. The presence of bacterial growth in the media after incubation was considered as a positive result.

Pot culture experiment

Pot culture was conducted to evaluate the effect of two selected *Pseudomonas aeruginosa* isolates PS 2 and PS 3 on control of chilli anthracnose under greenhouse conditions at School of Biosciences, MG University, Kottayam, Kerala, India. Chilli seeds of crop variety Ujwala were obtained from the Regional Agricultural Research Station, Kumarakom, Kottayam.

The properties of the soil were checked in the soil testing laboratory, Alappuzha. The soil (loamy texture, pH- 6.5; electrical conductivity- $0.1x10^{-3}$mhos/cm, organic carbon- 1.32%, organic nitrogen- 0.76 kg/ha, available phosphorus- 9.8 kg/ha, available potassium- 48.8 kg/ha, dehydrogenase activity- 0.75 µg TPF g^{-1}soil/day, phosphatase activity-3.25 µg PNPg^{-1}soil, bacterial total plate count-5.2×10^{6}cfu/ml, phosphate solubilizers-2×10^{6}cfu/ml) was air dried and passed through 2 mm sieve. The recommended dose of fertilizers (rock phosphate @ 60 kg $P_20_5ha^{-1}$, urea @ 75 kg N ha^{-1} and muriate of potash @ 25 kg K_20ha^{-1}) was used for each pot. Half of nitrogen, full phosphorus and half of potash were mixed with the soil and filled in earthen pots (30×30 cm) before transplanting at the rate of 10 kg per pot. One fourth of nitrogen and half of potash were applied 30 days after planting and remaining quantity of N was

applied two months after planting.

The experiments were conducted in a completely randomized design (CRD) with five treatments each having three replications. A standard culture of *Pseudomonas fluorescens* which can efficiently control chilli anthracnose was obtained from Tamil Nadu Agricultural University (TNAU) to compare the results with our test cultures. The bacterial isolates and the standard culture were inoculated into Kings B broth and incubated at 27°C for 2 days and 5ml of the resultant culture (5×10^9cfu/ml) was used for pot study. The pathogen used in the *in vivo* study, *C. capsici,* was isolated from the infected fruit collected from the Agricultural College Vellayani, Thiruvananthapuram. The pathogen was inoculated on oatmeal agar plates and incubated at 28±2°C for 5 days. The resultant culture was used for the induction of anthracnose disease in chilli plants under *in vivo* study.

Inoculation of Pseudomonas aeruginosa in greenhouse experiments

The chilli seeds were surface sterilized with 2% sodium hypochlorite for three minutes and then washed with sterile distilled water. The pots were then sown with sterilized chilli seeds (10 seeds/pot). Thinning was done after germination to retain four seedlings in each pot. After one month the chilli plants were subjected to foliar spray treatments. For foliar spray treatment, the plants were sprayed with 5 ml of 48 hrs old culture (5×10^9cfu/ml) of *Pseudomonas aeruginosa* Ps2, *Pseudomonas aeruginosa* Ps 3 and the standard *Pseudomonas* spp. Fresh seeds without any bacterial inoculum were used in control and fungicide treated treatments. Foliar application of the fungicide Mancozeb (0.2%) was also done after 30 days. A booster dose of cultures (5 ml of 48 hour old culture) was given after 30 days of first treatment. Plants were grown in greenhouse (average RH 78%, temperature 22 – 25 °C, natural day light) and watered regularly to maintain optimum soil moisture regime. The control plants did not receive any bacterial or fungicide treatment at any stage of growth. The pot experiment was conducted and repeated twice during 2014 – 2015 as Rabi crop.

Disease induction

Chilli anthracnose disease was artificially introduced by challenge inoculation of plants with *Colletotrichum capsici* on the 45th day of plant growth. Conidial suspension of *Colletotrichum capsici* was prepared in sterilized distilled water by harvesting acervuli from freshly sporulating cultures by scraping the surface of OMA slants with sterilized spatula. Serial dilutions of the spore suspension were prepared and inoculum density was adjusted to 5×10^5 spores /ml using a haemocytometer and the resultant fungal suspension was used to introduce anthracnose disease in the chilli plants according to the method described by Oh et al. (1999), with minor modifications. The control plants were inoculated with sterilized water.

The leaves and fruits of all the plants were subjected to examination of the development of anthracnose symptoms. Disease incidence was calculated according to the formula:

Percentage of disease incidence in leaves
= No. of leaves infected ÷ Total no. of leaves x 100

Percentage of disease incidence in fruits
= No. of fruits infected ÷ Total no. of fruits x 100

Effect of Pseudomonas aeruginosa in induction of defense enzymes against Colletotrichum capsici

The ability of the *Pseudomonas aeruginosa* isolates to induce systemic resistance in chilli plants was assessed under greenhouse conditions. Plants and plots were prepared as per the methods described for biocontrol study and three replications were maintained in each treatment. Four pots per replication were maintained. After 45 days, the plants were challenge inoculated with *Colletotrichum capsici*. The plants neither treated with biocontrol agents nor challenged with the pathogen were kept as control. The experiments were conducted using completely randomized design (CRD).

Chilli plants were carefully uprooted without causing any damage to root tissues at different time intervals (6, 12, 18 and 24 hours after the pathogen inoculation) and used for enzyme extraction. Four plants were sampled from each replication of the treatment separately and maintained for biochemical analysis. Leaf samples were washed under running tap water and homogenized with liquid nitrogen in a pre-chilled mortar and pestle and stored at 80^0 C for further studies.

Estimation of phenylalanine ammonia lyase (PAL) activity

Plant samples (1 g) were homogenized in 3ml of ice cold 0.1M sodium borate buffer, pH 7.0 containing 1.4 mM of 2-mercaptoethanol and 0.1 g of insoluble polyvinyl pyrrolidone. The extract was filtered through cheese cloth and the filtrate was centrifuged at 16,000 rpm for 15 minutes. The supernatant was used as enzyme source. PAL activity was determined as the rate of conversion of l-phenylalanine to trans- cinnamic acid at 290 nm (Dickerson et al., 1984). Samples containing 0.4 ml of enzyme extract were incubated with 0.5 ml of 0.1M borate buffer, pH 8.8 and 0.5 ml of 12 mM phenylalanine in the same buffer for 30 min at 30^0 C. The amount of *trans*- cinnamic acid synthesized was calculated (Dickerson et al., 1984). Enzyme activity was expressed as n mol *trans* - cinnamic acid $min^{-1}gm^{-1}$.

Assay of peroxidase (PO)

Leaf samples (1 g) were homogenized in 2 ml of 0.1M phosphate buffer, pH 7.0 at 4^0 C. The homogenate was centrifuged at 16,000 *g* at 4ºC for 15 min and the supernatant was used as enzyme source. The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1% H_2O_2. The reaction mixture was incubated at room temperature (28 ± 2ºC). The changes in absorbance at 420 nm were recorded. The enzyme activity was expressed as changes in the absorbance min^{-1} gm^{-1} (Hammerschmidt et al., 1982).

Assay of polyphenol oxidase (PPO)

Leaf samples (1 g) were homogenized in 2 ml of 0.1M sodium phosphate buffer (pH 6.5) and centrifuged at 16,000 *g* for 15 min at 4ºC. The supernatant was used as enzyme source. The reaction mixture consisted of 200 µl of the enzyme extract and 1.5 ml of 0.1M sodium phosphate buffer (pH 6.5). To start the reaction, 200 µl of 0.01M catechol was added and the activity was expressed as changes in absorbance at 495nm in $min^{-1}gm^{-1}$(Mayer et al., 1965).

Estimation of total phenolic content

Chilli leaf tissues (1 g) were homogenized in 10ml of 80 % aqueous methanol and agitated for 15 min at 70ºC. Methanolic extract (1 ml) was added to 5 ml of distilled water containing 250 µl of Folin–Ciocalteau reagent (1 N) and the solution was incubated at 25ºC. The absorbance of the blue colour developed was read using a spectrophotometer at 725 nm. Total phenolic content was calculated from standard graph drawn from catechol and Folin Ciocalteau reagent and expressed as catechol /g fresh weight.

Statistical analysis

The statistical analysis for all the parameters were performed using one way or two way analysis of variance. The means were compared by Duncan's Multiple Range Test using the statistical package SAS version 8.3 (SAS Institute Inc., Cary, NC, USA). The differences among the LS MEANS were analyzed by constructing diffogram (called the mean-mean scatter plot by Hsu, 1996). The diffogram produced by GLIMMIX is a two dimensional plot with both the vertical and horizontal axes of the same length and having the same numerical range defined by the difference between largest and smallest means (with a slightly larger range needed to plot the confidence intervals for the differences within the plotting area).

Results and Discussion

Dual culture assay

The antagonistic effect of the selected two bacterial isolates were tested against the chilli anthracnose pathogen *Colletotrichum capsici* by the standard dual culture method. Among the two isolates of *Pseudomonas aeruginosa,* Ps 2 showed maximum inhibition of 93.41% whereas the other isolate Ps 3 showed 72.5% of inhibition against *Colletotrichum capsici* after 5 days of incubation (Fig.1).

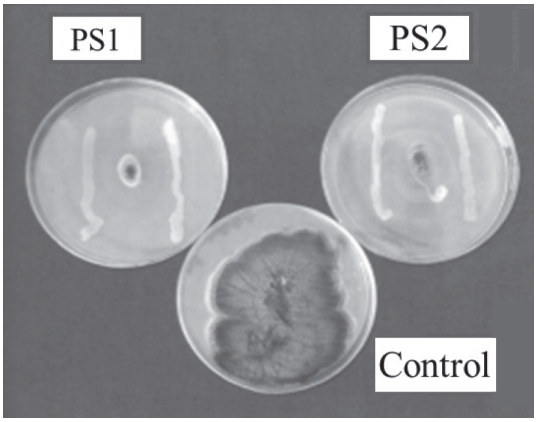


Figure 1. Antifungal activity *Pseudomonas aeruginosa* isolates against *Colletotrichum capsici*

Mechanism of antifungal activity

Both the isolates were capable of producing volatile organic compounds and thereby inhibiting the growth of the pathogen. Among the two isolates, Ps 2 showed maximum inhibition of 46.16% whereas Ps 3 showed inhibition of 40.34% against the growth of *Colletotrichum capsici* through the production of volatile organic compounds. Nonvolatile organic compound production showed a decline of 44.85% by Ps 2 against the growth of *Colletotrichum capsici* whereas Ps 3 showed 35.55 % of inhibition (Table 1).

Table 1. Growth reduction of *Colletotrichum capsici* by organic acid production

Isolate	% growth reduction of *Colletotrichum capsici*	
	Volatile organic acid*	Nonvolatile organic acid*
PS 2	46.16±0.28	44.85±0.78
PS 3	40.34±0.29	35.55±0.67

*Values are means of three replications ± SD

Both the isolates were capable of producing siderophore and HCN. The two isolates of *Pseudomonas aeruginosa* namely Ps 2 and 3 are positive towards ammonia formation. Ammonia production by the PGPB helps influence plant growth indirectly. Both the isolates are catalase positive because both of them showed the development of effervescence on addition of hydrogen peroxide to the bacterial culture. Among the two isolates Ps 2 is pectinase and chitinase positive but amylase, protease and lipase negative. The other isolate Ps 3 is amylase, pectinase and chitinase positive and protease and lipase negative. Both the isolates have the ability to produce salicylic acid. Production of salicylic acid by isolates 2 and 3 was found to be 11.3µg/ml and 14.1µg/ml, respectively. In the present study both the isolates showed positive results towards ACC deaminase production (Table 2).

Table 2. Secondary metabolite production and enzyme activity of isolates

Metabolite production*	Isolates	
	Ps 2	Ps 3
Siderophore	+ve	+ve
HCN	+ve	+ve
Ammonia	+ve	+ve
salicylic acid (SA)	+ve	+ve
ACC deaminase production	+ve	+ve
Chitinase	+ve	+ve
Amylase	-ve	+ve
Pectinase	+ve	+ve
Protease	-ve	-ve
Cellulase	-ve	-ve

*Mean of five replicates

The results of the *in vivo* antifungal activity of the selected *Pseudomonas aeruginosa* isolates *viz*., Ps 2 and 3 and the standard *Pseudomonas* culture obtained from TNAU were recorded after 90 days of pathogen inoculation when standard disease symptoms appeared both on the fruits and leaves (Tables 3 and 4). In control plant 100% of fruits and 80.66% of the leaves were infected by the

Table 3. *In vivo* antagonism of microbial antagonists *Pseudomonas aeruginosa* against anthracnose in chilli fruits

Sl.No.	Treatments	Total number of fruits**	Number of fruits infected**	% of disease incidence in fruits
1	Control	1	1	100
2	Fungicide (Mancozeb 0.2%)	2	0	0
3	*Pseudomonas aeruginosa* Ps 2	6	0	0
4	*Pseudomonas aeruginosa* Ps 3	7.66	0	0
5	*Pseudomonas fluorescens*	3.66	0.66	19.44

*All values are means from two repeated experiments with three replications each with 4 plants (4 plants/pot)
** Values are per treatment i.e., for all the plants in all replications

Table 4. In *vivo* antagonism of *Pseudomonas aeruginosa* against anthracnose in chilli leaves.

Sl.No.	Treatments	Total number of leaves**	Number of leaves infected**	% of disease incidence in leaves
1	Control	22.33 ^{d}	18 ^{a}	80.66
2	Fungicide (Mancozeb 0.2%)	34.33 ^{c}	1 ^{bc}	3.09
3	*Pseudomonas aeruginosa*PS 2	77.33 ^{b}	1.33 ^{bc}	1.70
4	*Pseudomonas aeruginosa*PS 3	91 ^{a} 0 ^{c}	0	
5	*Pseudomonas fluorescens*	73 ^{b} 3 ^{b}	4.11	

*All values are means from two repeated experiments with three replications each with 4plants (4 plants/pot) and Means in a column followed by the same letter(s) are not significantly different according to Duncan's Multiple Range Test (P=0.05) at 5% level of significance
** Values for all the plants in all replications

pathogen. Typical anthracnose symptoms appeared in the fruits and leaves of control plants which had not received any biocontrol and chemical fungicide treatment. In the case of fungicide treated plants, about 3.09 % of the leaves were infected, but disease development in fruits was zero. There was no disease incidence in the fruits of *Pseudomonas aeruginosa* Ps 2 and Ps 3 treated plants, but 19.44% of fruits were infected in standard treated plants. The leaves of *Pseudomonas aeruginosa* Ps 3 treated plants remained unaffected as where in *Pseudomonas aeruginosa* Ps 2 and standard treated plants 1.70 and 4.11% of the leaves were diseased (Figs. 2 & 3).

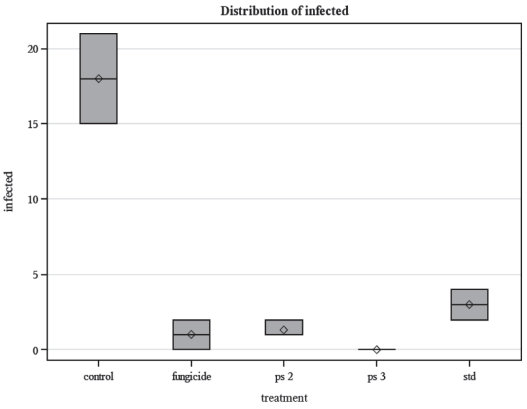


Figure 2. Induced disease suppression of anthracnose in chilli treated with distilled water as a negative control or Mancozeb 0.2% as a positive control or suspensions of *Pseudomonas aeruginosa* isolates *viz*., Ps 2 and 3, the standard *Pseudomonas* culture. All values are means from two repeated experiments with three replications each with 4 plants (4 plants/pot) under greenhouse experiments.

The popularity and demand for chilli are providing a boost to the chilli industry, but production is increasingly constrained by the prevalence of plant diseases. Among the diseases, anthracnose caused by *Colletotrichum capsici* is the most important disease. Under suitable weather conditions, the disease may cause 12-15% loss in the crop (Suthin et al., 2006; Suthin and John, 2008). In the present study two *Pseudomonas aeruginosa* isolates namely Ps 2 and 3 showed significant activity against anthracnose fungi *Colletotrichum capsici* under *in vitro* condition. In a study carried out by Shilpa and Gokulapalan (2015), *T. viride* caused 55.5%

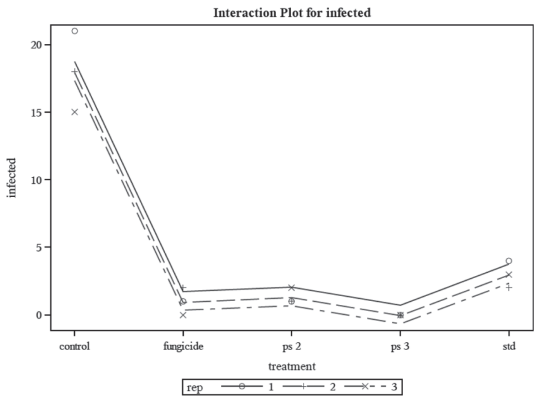


Figure 3. Interaction plot for disease incidence in leaves of different treatments under greenhouse experiments.This plot confirms the results of the F test on whether or not the interaction is significant. The points for each appraiser average measurement per part are connected to form k (number of appraisers) lines. The way to interpret the graph is if the k lines are parallel there is no interaction term. When the lines are nonparallel, the interaction can be significant. Here the lines are nearly parallel, indicating no significant interaction. All values are means from two repeated experiments with three replications each with 4 plants (4 plants/pot)

mycelial growth inhibition of *Colletotrichum capsici* in dual culture. In the same study *Pseudomonas flourescens* showed 90% of the radial growth inhibition of *Colletotrichum capsici*. Similarly Kaur et al (2006) noticed 53.0% inhibition of *C.capsici* by *T. viride* and complete overgrowth by the biocontrol agent. The formation of hyphal coils by *T. viride* on pathogenic colonies was also noticed. Of the various rhizospheric bacteria, the bacteria belonging to *Pseudomonas*, which colonize roots of a wide range of crop plants, are reported to be antagonistic to soil borne plant pathogens (Ehteshamul-Haque et al., 2007; Siddiqui et al., 2000). The production of certain antibiotics (Levy et al., 1992) and siderophores (De Meyer and Hofte, 1997; Buysens et al., 1996) by *Pseudomonas aeruginosa* has been regarded as one of the mechanisms involved in antagonism. In the present investigation, the two isolates tested are siderophore and HCN positive. The iron concentration in the soil is low enough (10-7M) to limit the growth of soil microorganisms (10-8 – 10-6 M) (Kloepper et al., 2004). Rhizobacteria have to develop some strategies to acquire iron. The major strategy is the production and utilization of siderophores. The rhizobacteria that can produce siderophores could compete for iron with soil borne pathogens. Competition for iron is also a possible mechanism in agriculture to control the pathogenic fungi in the soil. HCN, a volatile metabolite is thought to play a major role in biological control of some soil borne diseases (Siddiqui et al*.,* 2006). In pseudomonad species, HCN is released by the decarboxylation of glycine (Wissing, 1975). Volatile and non volatile organic acid production is also shown by the isolates employed in the present study. Microbial degrading enzymes of the cell wall of fungal pathogens have been reported (Fridlender et al., 1999). Bacterial strains showing catalase activity must be highly resistant to environmental, mechanical and chemical stress (Joseph et al., 2007). Here Ps 2 is pectinase positive, whereas Ps 3 is amylase and pectinase positive. The presence of these enzymes may be the reason for the antifungal activity shown by these isolates. The two isolates also produced salicylic acid at a significant level, which may contribute to the disease suppression ability of the isolates. Hamada and Hashem (2003) found that soaking wheat grains in SA before sowing significantly reduced the values of mean disease rating caused by *Bipolaris sorokiniana, F. oxysporum* or *F. graminearum*. It was also reported that SA induced plant resistance against pathogens and stimulated plant growth (Vidhyasekaran, 1990).

The results of the greenhouse study showed that the selected *Pseudomonas aeruginosa* isolates were excellent biocontrol agents, since they exhibited significant levels of antifungal activity against *Colletotrichum capsici*. There are several studies on biocontrol of chilli diseases by rhizosphere microbes (Rini and Sulochana, 2006; Vasanthakumari and Shivanna, 2013; Sudhir et al., 2014). Suthin and John (2008) reported the ability of *Trichoderma harzianum* to reduce *Colletotrichum capsici* fruit rot in chilli. Sudhir et al., (2014) reported the control

of chilli anthracnose by endophytic *Pseudomonas aeruginosa* isolated from chilli fruits. Majority of antagonistic bacteria are Gram negative and belong to the group of fluorescent pseudomonads, which are efficient biocontrol agents (Bloemberg and Lugtenberg, 2001). Antagonistic activities were also reported in *Pseudomonas chlororaphis, P. fluorescens, P. graminis, P. putida, P. tolaasii* and *P. veronii* (Adhikari et al., 2001).

Induction of systemic resistance by Pseudomonas aeruginosa against Colletotrichum capsici under greenhouse conditions

Activation of the plant's own defense system with the aid of biotic and abiotic inducer is a novel technology in the management of plant diseases. Microbial products have been considered as one of the major groups of compounds that induce systemic resistance. Biologically active compounds present in biocontrol agents act as elicitors and induce resistance in host plants resulting in reduction of disease development. PGPR induced systemic resistance (ISR) is similar to pathogen induced systemic acquired resistance (SAR) in non-infected parts and made plants more resistant to pathogen infection and are effective against a wide range of foliar and root pathogens (Zhang et al., 2002)

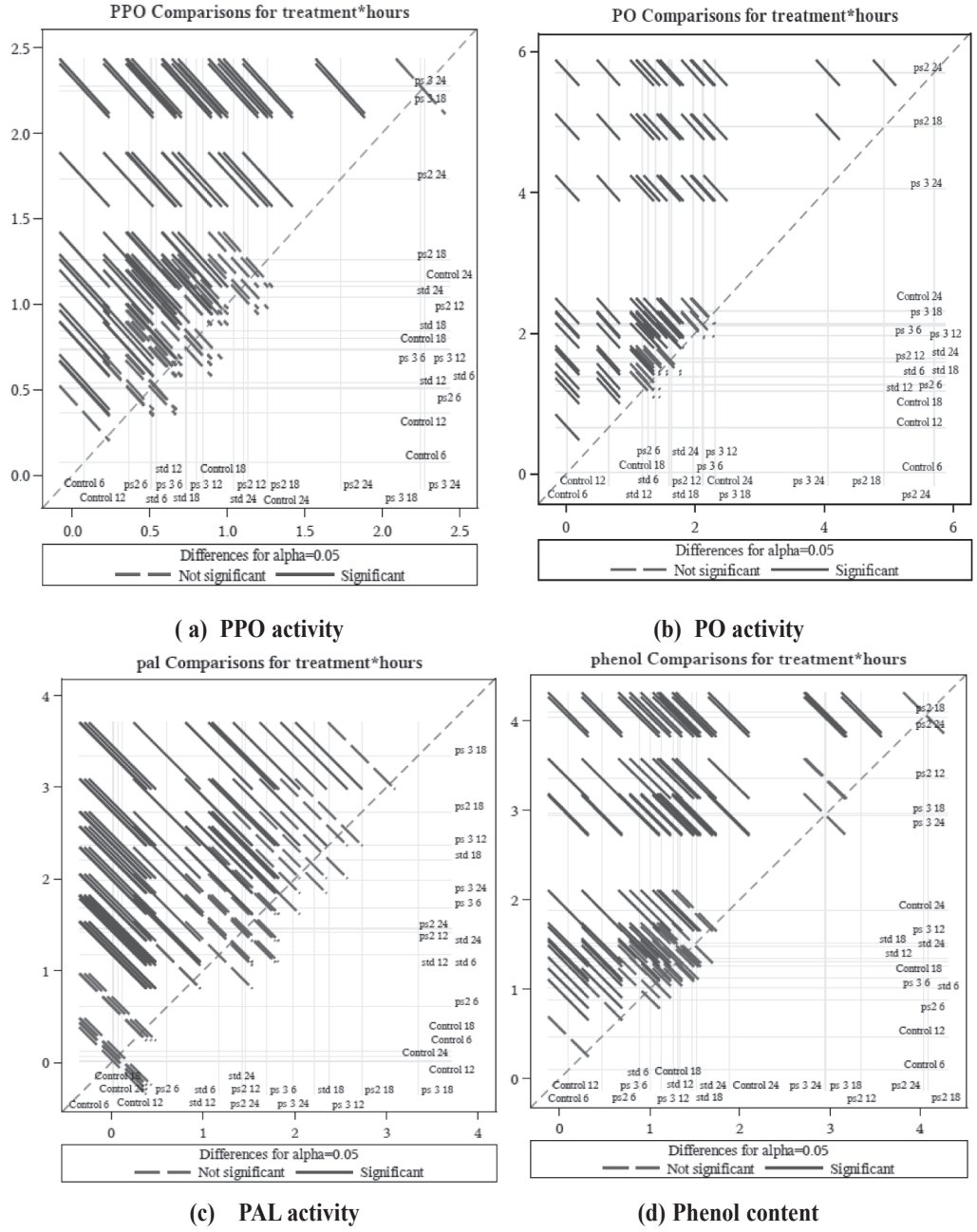
Phenylalanine ammonia lyase is the first key enzyme in the phenyl propanoid metabolism and plays a significant role in the regulation of biosynthesis of phenols in plants (Lawton and Lamb, 1987). PAL catalyzes the conversion of phenylalanine to trans-cinnamic acid, which supplies the precursors for flavonoid pigments, lignins and phytoalexins (Hahlbrock and Scheel, 1989). The activation of PAL and subsequent increase in phenolic content in plants is a general response associated with disease resistance (Velazhahan and Vidhyasekaran, 1999). Inhibition of PAL affects subsequent pathways of phenolic compound synthesis.

Peroxidases are involved in phenyl propanoid metabolism, regulation of plant cell elongation, phenol oxidation, polysaccharide cross-linking, IAA oxidation, cross-linking of monomers, oxidation of hydroxyl cinnamyl alcohols into free radical intermediates and wound healing (Vidhyasekaran et al., 1997). Plant peroxidases are heme - proteins that use H_2O_2 to oxidize a large variety of hydrogen donors such as phenolic substances, amines, ascorbic acid, indole and certain inorganic ions (Van Huystee, 1987). In the present study, a significant increase in peroxidase activity was noticed after 6 hrs of treatment and continued upto 24 hrs of incubation in all the treatments including the control plant (Fig. 4).

Polyphenol oxidase is a copper containing enzyme, which oxidizes phenolics to highly toxic quinones and is involved in the terminal oxidation of diseased plant tissues, which attributes for its role in disease resistance. In the present study, polyphenol oxidase activity was comparatively high in bacteria treated leaf extracts as compared with the control. There was a significant increase in the PPO level with the increase in time (Fig. 4). The increase in polyphenol oxidase activity might be due to activation of latent host enzyme, solubilisation of host polyphenol which was normally particulate or even due to *de novo* synthesis (Rao et al., 1988).

Phenolic compounds act as anti microbials (Jung et al., 2004) and are critical to host defense in sensing and defense-triggering in host pathogen interaction (Beckman, 2000). Studies have demonstrated rapid esterification of phenolic compounds into the plant cell wall as a common and early response in the expression of resistance (Nicholson and Hammerschmidt, 1992). In the present study, phenolic content was comparatively high in bacteria treated plants compared to the untreated control.

The ability of the selected *Pseudomonas aeruginosa* isolates to control chilli anthracnose was proved under greenhouse studies. Management of plant diseases by the application of biocontrol agents provides one component in integrated disease



(a) PPO activity **(b) PO activity**

(c) PAL activity **(d) Phenol content**

Fig. 4. Diffogram of of defense enzyme production induced by *Pseudomonas aeruginosa* in chilli against *Colletotrichum capsici*.under green house experiment. .A significant difference between pairs of ls means(i.e., the adjusted pvalue is less than 0.05) occurs when the adjusted lower and upper endpoints of the confidence intervals are both positive or both negative; that is, the solid lines sloping at -45 degrees fall completely above or below the line of equality. This line of equality also identifies non-significant differences; when the line for the confidence interval intersects the diagonal (shown with a dashed line), the value 0 is included within the confidence interval. The attributes are (a) polyphenol oxidase (PPO) (b) peroxidase (PO) (c) phenylalanine ammonia lyase (PAL) (d) total phenolic content.

management. Induction of defense enzymes *viz,* Peroxidase (PO), Polyphenol oxidase (PPO) and Phenylalanine ammonia lyase (PAL), is one of the key factors in the suppression of pathogen and disease development. Here the two *Pseudomonas aeruginosa* isolates induced systemic resistance in host plants. Hence, these biocontrol agents are capable of reducing fungal disease in chilli and will also help to reduce the usage of chemical fungicides which will adversely affect the environment.

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