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Full Length Research Paper

Screening of *Pseudomonas fluorescens* isolates for biological control of *Macrophomina phaseolina* root-rot of safflower

Govindappa, M.[1]*, Ravishankar, Rai V.[2] and Lokesh, S.[3]

[1]Department of Biotechnology, Shridevi Institute of Engineering and Technology, Sira Road, Tumkur-572 106, India.
[2]Department of Studies in Microbiology, University of Myosre, Manasagangotri, Mysore-570 006, India.
[3]Department of Studies in Applied Botany, Seed Pathology and Biotechnology, University of Myosre, Manasagangotri, Mysore-570 006, India.

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The aim of this study was to assess the potential bacterial antagonists to control *Macrophomina phaseolina* root-rot of safflower by using screening methods. In total, 38 fluorescent pseudomonads isolated from rhizosphere soil of healthy safflower plants were collected from major safflower growing areas, 13 were initially selected based on dual culture technique on potato dextrose agar media. Furthermore, these13 isolates were screened for reduction of percent incidence of *M. phaseolina* and to check their efficacy in seed germination and seedling growth in laboratory condition. Most of the isolates showed varying levels of antagonism against *M. phaseolina* in both standard blotter method and paper towel method. In addition, few isolates increased percent germination, root-shoot lengths of safflower. Among them, CTPF31 showed the highest ability to reduce the root-rot disease severity in the greenhouse biological control test. The promised fluorescent pseudomonads (CTPF31 and CTPf36) enhanced the defense enzymes, peroxidase, PAL, β 1,3-glucanase, and chitinase after challenge inoculation with the target pathogen. These results indicated that the dual culture technique and seedling assay are more important for selection of promising biocontrol agents against *M. phaseolina,* besides they also increased the plant growth by controlling the disease as evidenced by induction of defense enzymes.

Key words: Plant growth-promoting Rhizobacteria, safflower, root-rot, *Macrophomina phaseolina*, Pseudomonas fluorescens*,* biological control.

INTRODUCTION

Safflower (*Carthamus tinctorius* L.) is an important oil seed crop in India. It suffers severely from seed-borne fungal diseases, which may appear either at the seedling stage, flowering stage or at the time of seed formation, causing heavy yield and quality losses. Root-rot caused by *Macrophomina phaseolina* (Tassi.) Goidanich, which is a serious menace in Karnataka, Indian may kill up to 20-25% of plants in commercial fields (Singh and Bhowmikh, 1979). Application of synthetic pesticides in the agricultural field results in their persistence for quite a long time and is usually not desired. Some fungicides simultaneously offered protection against fungi through seed treatment, but it may not enhance the quality to a desired extent and chemical residues are major problems leading to environmental pollution and human health hazards. Thus, alternative control measures for the control of *Macrophomina* root-rot should be developed. Biological control is proposed to be an effective, safe and ecologically friendly approach for plant disease management. Bio-pesticides are up to 50% cheaper than synthetic pesticides.

They are eco-friendly in nature, have a high cost-benefit ratio and do not pose the risk of the pathogen developing resistance. They are easy to apply and are compatible with bio fertilizers. *Pseudomonas* spp. has been used extensively as biological control agents against wide spectrum of fungal pathogens in several

*Corresponding author. E-mail: dravidateja07@yahoo.co.in. Tel: +91-9686114847, +91-816-2212626. Fax: +91-816-2212629.

crops (Van Peer et al., 1991; Nielsen et al., 1998; Kavitha et al., 2005; Vidhyasekaran and Muthamilan, 1999). Mechanisms involved in disease suppression by *Pseudomonas* spp. involve in the production of siderophores (Klopper et al., 1980; Becker and Cook, 1988; Loper, 1988), hydrogen cyanide (Alh et al., 1986), lytic enzymes (Frindlender et al., 1993; Sneh et al., 1984), the biosynthesis of antibiotics (Howie and Suslow, 1991) and competition for nutrients and space (Elad and Chet,1987). *Pseudomonas fluorescens* are known to promote plant growth by producing gibberllins, cutokinin and indole acetic acid (Dubeikovsky et al., 1993) and also act as inducers of systemic resistance in plants (Alstrom, 1991; Van Peer et al., 1991; Wei et al., 1991, Zhou and Paulitz, 1994; Raupach et al., 1996; Pieterse et al., 2001). A variety of soil micro-organisms have been demonstrated in the control of fungal plant pathogens including *M. phaseolina*. *Trichoderma harzianum*, *Bacillus subtilis* and *P. fluorescens* have been used as biological control agents against *M. pahseolina* in French bean and chick pea (Mukherjee and Tripathi, 2000; Selvarajan and Jayarajan, 1996). The literature survey shows there is no evidence of using *P. fluorescens* in the control of root-rot disease in safflower. The problem of identifying an effective biological control of *M. phaseolina* is the screening method used against *in vitro* conditions, correspondingly, *in vivo* efficacy and estimation of defense enzymes (Weller and Cook, 1983; Reddy et al., 1993). Ideally, the candidate organisms should be screened on plants rather than *in vitro* (Weller, 1988). However, it is expensive and time consuming to conduct large scale screening tests as whole plants. As a compromise, it is required to develop an efficient *in vitro* screening method which allows identifying potential antagonists for plants in greenhouse and field conditions. Therefore, in this study fluorescent pseudomondas isolated from rhizosphere soil of safflower (major growing areas of Karnataka, India) were screened to identify new potential biocontrol agents against *M.phaseolina* in safflower using dual culture technique and seedling assay. A large number of enzymes have been associated with induced systemic resistance (ISR), including peroxidase, phenylalanine ammonia-lyase, β-1,3-glucanase and chitinase show synergic antifungal activity and are related to the systemic acquired resistance pathway that includes salicylic acid as signal molecule (van Loon et al., 1994). Based on this, there were made attempts to detect increases in activity of enzymes known to be involved with induced resistance such as peroxidase, PAL, β1,3-glucanase, chitinase and phenolic substance.

MATERIALS AND METHODS

Culture media and organisms

Fluorescent pseudomonads were isolated from the healthy rhizosphere soils of safflower growing areas of Karnataka, India using King's B medium (KBM) by serial dilution method (Crossan, 1967; King et al., 1954). After performing 10 fold dilutions, 100 µl aliquots were plated on KBM and incubated at 28±2℃ for 48 h before colonies were isolated. Fluorescence of the colonies under ultra violet (UV) light was taken as primary criterion for selection of the isolates. All isolates were grouped according to the place, size and morphology of the colonies on the medium and maintained on KBM slants at 4℃. A virulent strain of *M. phaseolina* was isolated from an infected safflower plant maintained on potato dextrose (PDA) medium.

Dual culture technique

All the bacteria isolates isolated were screened for their ability to inhibit the growth of *M. phaseolina* on PDA plates using dual culture technique (Yoshida et al., 2001). Mycelial plug of *M. phaseolina* (1 cm diameter) was placed at the center of the agar medium in a 9 cm Petri plates. Four spots were made on the edge of the plate with an actively growing suspension of the bacterial isolate after 48 h of fungal inoculation. Plates were inoculated for 6 to 8 days at 24±2℃ and the inhibition of fungal growth was assessed by measuring the diameter of the inhibition zone (in cm).

Selection of seed sample and mass culture of *P. fluorescens*

Highly infected variety Manjira were collected for further study from Safflower Research Station, Annigeri, University of Agriculture, Dharwad, Karnataka, India. Thirteen different isolates of *P. fluorescens* were cultured on KMB at 30℃. Two-day-old cultures were harvested and centrifuged at 10,000 rpm for 10 min. Pellets obtained were suspended in distilled water and the inoculum load was adjusted to 1×10^8 CFU/ml based on spectrophotometry (OD 0.45 at 610 nm). The seeds were separately soaked overnight in the culture suspension and air-dried. Control seeds were soaked in distilled water.

Effect of *P. fluorescens* on the incidence of *M. phaseolina* and seedling growth

The most promising isolates of *P. fluorescens* were selected for the seed treatment. Treated seeds were plated equidistantly on three layers of wet blotters on Perspex plates and incubated at 22±2℃ under alternate cycles of 12/12 h near UV light and darkness for 7 days. On the 8th day of incubation the seeds were evaluated for the pycnidia of *M. phaseolina* following the procedures of international seed testing association, ISTA (ISTA, 1996). Treated and untreated seeds were also subjected to the paper towel method, in which the seeds were plated equidistantly between wet blotters and incubated at 22±2℃ under 12/12 h alternate cycles of light an d darkness for 14 days. After incubation, the seed germination percentage and root-shoot lengths of the seedlings were assessed and the vigour index was calculated based on the formula prescribed by Abdul Baki and Anderson (1973).

In vivo biological control assay

Based on the aforementioned *in vitro* screening tests, 5 isolates (CTPf9, CTPf10, CTPf31, CTPf 23 and CTPf36) were selected for further *in vivo* biological control studies. A suspension of individual antagonistic isolates was prepared in sterile water as described earlier. Seeds of safflower were surface-sterilized and inoculated by soaking them over-night in a bacterial suspension containing 1×10^8 CFU/ml. The seeds were in sterile water which served as control. The inoculated and un-inoculated seeds were plated in 25 cm

diameter earthen pots with soil mixtures (soil: sand: farmyard in the ratio of 1:1:1) and incubated in a greenhouse at 22±2℃. Seedlings at four leaf stage were inoculated with *M. phaseolina* by adding 2 ml of mycelial suspension in sterile distilled water ($18x10^6$ mycelial fragments/ml) at the base of each seedling. Controls were treated with only sterile distilled water. The percent disease incidence, disease severity and plant height were recorded on the 40^{th} day after sowing. Disease severity was assessed using a 0-5 scale where '0' represents no visible disease symptoms, '1' represents leaves slightly yellowing, black necrotic lesions, root-rot and collar rot lesions beginning to appear on stem, '2' represents 30-50% of entire plant diseased, '3' represents 50-70% of entire plant diseased, '4' represents 70-90% of entire plant diseased and '5' represents plant dead. Plant growth was measured by randomly selecting 5 plants on each pot. One hundred (100) seeds were maintained for each treatment.

Estimation of defense enzymes

Leaf samples were collected from different isolates of *P. fluorescens* treated samples from the greenhouse as described earlier. Leaf samples were collected after a challenge inoculation with the target pathogen *M. phaseolina* at different intervals of the time (2, 4, 6 and 8 days after challenge inoculation).

Peroxidase assay

Fresh plant leaves (1 g) homogenized in 3 ml of 0.1 M sodium phosphate buffer (pH 7.0) with pre-chilled mortar and pestle. The homogenate were centrifuged at 18,000 rpm at 5℃ for 1 5 min and used within 2-4 h. A supernatant was served as an enzyme source. To a spectrophotometric sample cuvette, 3 ml of buffer solution, 0.05 ml guaiacol solution, 0.1 ml enzyme extract and 0.03 ml hydrogen peroxide (H_2O_2) solution were added and mixed well. Absorbance was recorded at 470 nm using spectrophotometer (Hitachi, 2000). The enzyme activity was expressed as changes in absorbance min^{-1} g^{-1} fresh weight (Hammerschmidt et al., 1982). Three replicates were maintained for each treatment.

Determination of phenylalanine ammonia lyase (PAL) activity

Leaf tissues (300 mg) from each of the three replicates for each treatment were homogenized in the ice-cold 0.25 M borate buffer (pH 8.7) in an ice bath. The homogenate was centrifuged at 5000 rpm for 15 min at 4℃. The supernatant was then centri fuged at 15000 rpm for 15 min at 4℃. The resultant clear yell owish-green supernatant which contained 1 ml of enzyme extract, 0.5 ml of 0.2 M borate buffer (pH 8.7), 1.3 ml of distilled water and 0.2 ml of 1 M l-phenylalanine, was used as crude enzyme extract. Changes in absorbance at 290 nm were observed using spectrophotometer (Hitachi, 2000). Reaction mixtures without substrate served as control. One unit of enzyme activity produced 3.37 nm of cinnamic acid/h (Singh and Prithiviraj, 1997). Three replicates were maintained for each treatment.

β-1,3-Glucanase assay

The crude extracts of 62.5 µl was added to 62.5 µl of lamanarin and then incubated at 40℃ for 10 min and the reaction w as stopped by adding 375 µl of dinitrosalicylic acid and heated for 5 min on boiling water bath. The resulting solution was diluted with 4.5 ml distilled water and the absorbance was read at 500 nm. The crude extract preparation with lamanarin with zero time incubation served as blank. The activity was expressed as µg equivalent of glucose/ min/mg of protein (Kavitha et al., 2005).

Chitinase assay

One gram (1 g) of sample was ground using a chilled pestle and mortar with 0.1 M sodium citrate buffer (pH 5.0) at 4℃. The homogenate was centrifuged at 10,000 rpm for 20 min. The supernatant was used as a crude enzyme extract for assaying chitinase activity. The changes in the chitinase activity were determined by colorimetric assays described by Boller and Mauch (1988).

Estimation of phenolic substances

One gram of fresh sample was homogenized with 10 ml of 80% methanol and agitated for 15 ml at 70℃. One millili ter of the methanolic extract was added to 5 ml of distilled water and 250 µl of Folin-Ciocalteau reagent (1 N) and the solution was kept at 25℃. The absorbance of the blue was measured using a spectrophotometer (Hitachi, 2000) at 725 nm. Catachol was used as the standard (Kagale et al., 2004).

Statistical analysis

Percentage data were transformed to arcsine values and the analysis of variance was carried out (ANOVA). Means were compared for significance using Duncan's new multiple range test (DMRT; p=0.05). All experiments were repeated at least three times. Since repeated tests yielded similar results, data from a single representative experiment are presented. Variations in absolute results among the greenhouse experiments may have been the results of seasonal effects.

RESULTS

Dual culture techniques

Thirty-eight (38) isolates from the rhizosphere soil of safflower were classified as *P. fluorescens* and the soil samples were collected from different places of Karnataka, India. The *P. fluorescens* isolates were fluorescent on KBM, Gram negative, oxidase and catalase positive. Table 1 shows the results of *in vitro* screening of *P. fluorescens* against *M. phaseolina* using dual culture technique. Inhibition was clearly discerned by limited growth or by the complete absence of fungal mycelium in the inhibition zone surrounded by bacterial colony. Among the 38 isolates tested, 13 showed inhibitory effect on the growth of *M. phaseolina*. Among these 13 isolate, five exhibited maximum inhibition of mycelial growth by recording the inhibition zone of CTPf31 and CTPf36 respectively.

Effect of *P. fluorescens* on the incidence of *M. phaseolina* and seedling growth

Table 2 reveals the percentage incidence of *M. phaseolina*

Table 1. Inhibition of *M. phaseolina* by *P. fluorescens* isolates in dual culture technique.

P. fluorescens isolate	Zone of inhibition in PDA
CTPf6	+
CTPf9	++
CTPf10	++
CTPf13	+
CTPf16	++
CTPf19	++
CTPf21	++
CTPf23	+
CTPf26	++
CTPf30	+
CTPf31	+
CTPf34	++
CTPf36	++

Data are based on four replicates of each experiment. + represents 1-5 mm wide zone; ++ represents 6-10 wide zone.

Table 2 . Effect of *P. fluorescens* isolates on percent incidence of pathogen and seed germination as well as seedling vigour in standard method and paper towel method, respectively.

P. fluorescens isolate	Incidence of *M. phaseolina* (%)	Germination (%)	MRL	MSL	Vigour index
CTPf6	11	89	9.1 ± 1.4^{gh}	7.2 ± 0.9^{fg}	1451
CTPf9	8	90	9.8 ± 1.2^{de}	7.8 ± 1.4^{cd}	1584
CTPf10	8	90	10.1 ± 1.3^{cd}	7.7 ± 0.6^{de}	1602
CTPf13	10	90	9.3 ± 0.6^{fg}	7.1 ± 0.9^{fg}	1476
CTPf16	10	90	9.6 ± 0.4^{ef}	7.4 ± 1.1^{ef}	1530
CTPf19	12	90	9.8 ± 0.7^{de}	8.1 ± 0.8^{bc}	1611
CTPf21	9	91	10.2 ± 0.1^{cd}	7.9 ± 1.1^{cd}	1347
CTPf23	13	90	9.7 ± 1.3^{de}	7.7 ± 1.1^{de}	1566
CTPf26	7	93	10.8 ± 0.6^{bc}	8.1 ± 0.8^{bc}	1758
CTPf30	14	90	9.5 ± 0.9^{ef}	7.4 ± 0.6^{ef}	1521
CTPf31	13	90	9.8 ± 1.2^{de}	7.5 ± 0.4^{ef}	1557
CTPf34	2	94	11.6 ± 0.4^{a}	8.9 ± 1.4^{a}	1927
CTPf36	5	93	11.2 ± 0.8^{ab}	8.4 ± 1.1^{ab}	1823
Control	16	78	8.5 ± 1.3^{i}	6.9 ± 0.9^{h}	1201

Data based on four replicates of 100 seeds, MRL, mean root length; MSL, mean shoot length, ± standard error.

in the seeds of safflower variety Manjira due to different isolates of *P. fluorescens*. Among the 13 isolates used for seed treatment, 5 of them (CTPf9, CTPf10, CTPf31, CTPf 23 and CTPf36) showed the broadest range of inhibition of the pathogen on seeds. Isolate CTPf31 was found to be effective against *M. phaseolina*. Isolates CTPf23, CTPf9 and CTPf10 were found to be least effective against this pathogen. Table 2 also illustrates that all the *P. fluorescens* isolates tested possessed phytostimulatory properties which improved seed germination and seedling vigour, the best results being recorded for isolates CTPf9, CTPf10, CTP31, CTPf23 and CTPf36 which enhanced the seedling germination by 12-16% over control.

Greenhouse studies

The greenhouse efficacy of selected *P. fluorescens* isolates (CTPf9, CTPf10, CTPf31, CTPf23 and CTPf36) for the control of *Macrophomina* root-rot in safflower plants was evaluated under greenhouse conditions

Table 3. Effect of antagonistic *P. fluorescens* isolates on *M. phaseolina* root-rot disease development and safflower growth in greenhouse conditions.

***P. fluorescens* isolate**	**Percent disease**	**Plant height (with *M. phaseolina*) (cm)**
CTPf9	18.45±3.11^{b}	13.79±0.33^{de}
CTPf10	12.36±1.57^{c}	14.19±0.13^{cd}
CTPf31	2.63±1.21^{f}	16.36±0.52^{a}
CTPf136	4.41±1.7^{e}	15.47±0.84^{b}
CTPf23	8.73±1.53^{d}	15.21±0.71^{bc}
Control	21.24+3.24^{a}	11.98+0.67^{f}

Data was based on 100 plants for each treatment. Disease severity was assessed on 0-5 scale from, 0 (no visible disease symptoms) to 5 (plant death). ± Standard error, according to Duncan's Multiple Range Test (DMRT), values followed by different superscripts are significantly different at p≤0.05.

(Table 3). The *M. phaseolina* inoculated and un-inoculated plants, symptoms of root-rot initially appeared by gradual yellowing and drying of leaves, when the plants were mostly in the flowering stage. The yellowing started from the lower portion of the plant and gradually extended upwards, while the terminal leaves and flower buds remained green for some time. The affected roots exhibited extensive shredding of the black tissue and stem splitting. The split area of the stem was hallow and brown with light white to grey mycelial mats adhering to the inner surface accompanied by a wilt due to root-rot of the entire plants, leaf defoliation and damping-off. The plants treated with antagonistic *P. fluorescens* isolates showed much variation in the reduction of the disease severity. Although, isolates CTPf9 and CTPf10 showed high inhibition in the dual culture assay, they failed to reduce the disease severity and disease incidence in the greenhouse assay. The highest reduction of the disease incidence and disease severity was observed when the seeds were treated with isolate CTPf31. The average disease incidence was observed in the plants treated with isolate CTPf23 when compared with *M. phaseolina* treated control. The antagonistic *P. fluorescens* isolate-treated plants showed plant growth promotion, monitored by measuring the plant height revealed a wide variation between treated and untreated plants. *M. phaseolina* treated plant resulted in disease in the plant growth compared to control and this effect was suppressed when the plants were treated with the isolates. After the challenge inoculation with *M. phaseolina*, the highest effect was observed with isolate CTPf31 which enhanced the plant growth by 5.3% compared to un-treated control. In the presence of *M. phaseolina*, isolate CTPf23, CTPf9 and CTPf10 significantly increased the plant growth by 3.5, 1.8 and 2.2%, respectively when compared with the control. Isolate CTPf9 did not influence the growth of plants in the presence of *M. phaseolina*.

Estimation of defense enzymes against root-rot in *P. fluorescens* treated plants

Peroxidase assay indicated an increased peroxidase activity in the seedlings inoculated with the target fungus. After 6 days challenge inoculation, the seedlings showed highest activity and gradual persistence of resistance (Figure 1). Isolate CTPf31 and CTPf36 were very effective in the enhancement of peroxidase when compared with other strains (CTPf9, CTPf10 and CTPf23) (Figure 2). It can be seen that micro-mobilization of seeds with different isolates of *P.fluorescens* lead to an increase in PAL activity. Also, changes in PAL activities were observed after the challenge inoculation with the target pathogen up to 8 days. CTPf31 and CTPf36 were most effective in enhancing the PAL activity compared to other isolates (CTPf9, CTPf10 and CTPf23). The β 1, 3-glucanase activities were increased 6 days after challenge inoculation. CTPf31 and CTPf36 pretreated seedlings enhance their β1,3-glucanase activity compared with other isolates (CTPf9, CTPf10 and CTPf23) (Figure 3). The activity reached maximum at day 6, after the challenge inoculation and it was decreased in day 8. Increase in chitinase activities were observed in CTPf31 and CTPf36 pre-treated seedlings compared with other isolates (CTPf10, CTPf9 and CTPf23) (Figure 4). Pre-treated safflower plants which were challenge-inoculated with the pathogen, remained at higher level on days 6 after challenge inoculation and decreased in day8. The activity reached maximum levels on the 6th day after challenge inoculation. Phenolic compounds may be fungitoxic in nature and may increase the mechanical strength of the host cell wall. In this study, seed treatment with CTPf31, CTPf36 and CTPf10 resulted in increased accumulation of phenolic substances in response to pathogenic infection by the pathogen (Figure 5). Higher level of phenol activity was observed in these isolates treated seedlings compared with other isolates (CTPf9 and CTPf23).

DISCUSSION

Plant growth promoting rhizobacteria are free living bacteria having a beneficial effect on plants as they enhance emergence, colonize roots and stimulate growth

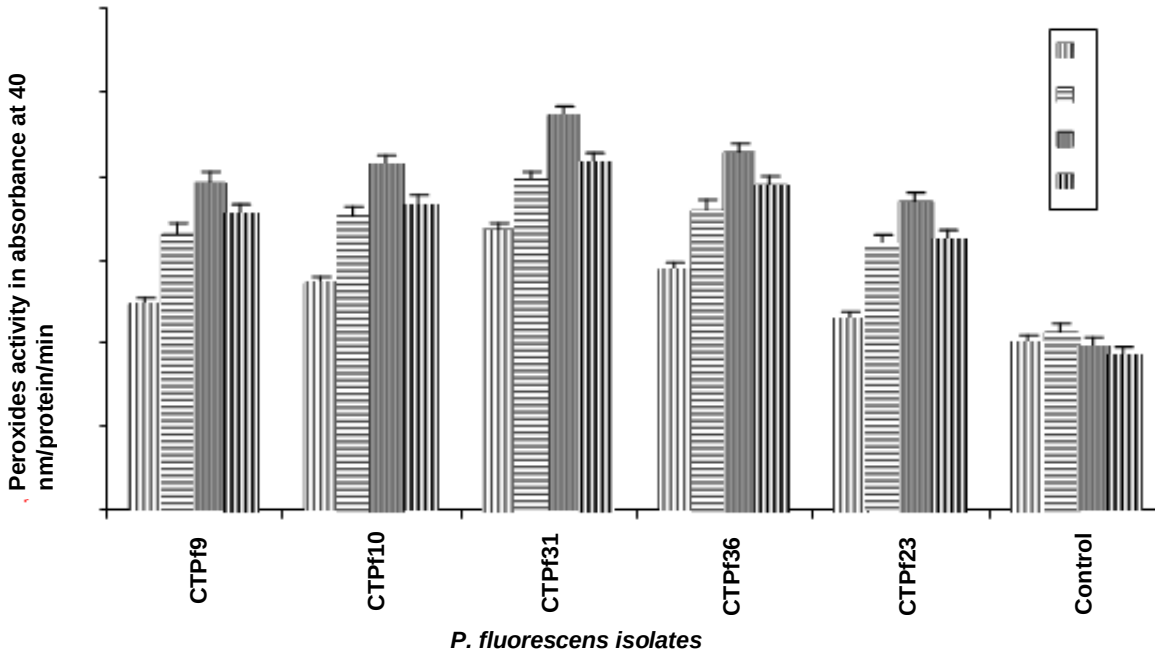

Figure 1. Effect of seed treatments with *P. fluorescens* on peroxidase activity in safflower plants after challenge inoculation with *M. phaseolina*.

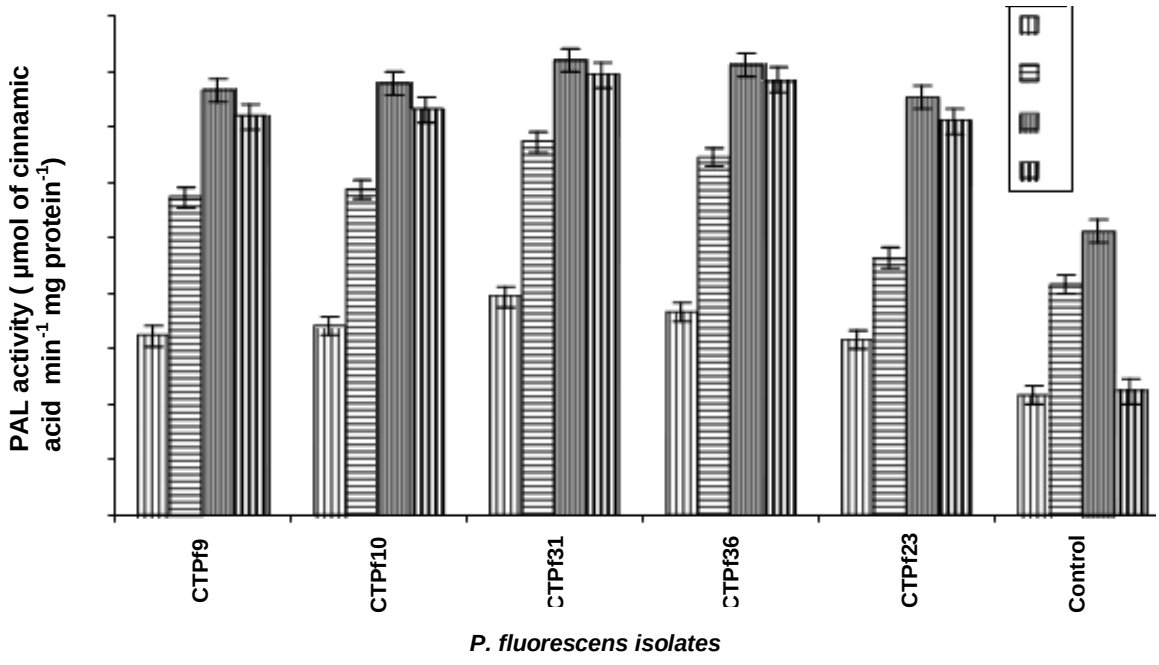

Figure 2. Effect of different isolates of *P. fluorescens* on PAL activity in safflower plants after challenge inoculation with *M. phaseolina*.

(Kloepper et al., 1988). They also control many fungal pathogens when used as seed treatment (Hoflich et al., 1994; Wei et al., 1996; Vidhyasekaran and Muthamilan, 1999; Rajkumar et al., 2005). In this study, root associated bacteria were isolated from the rhizosphere of safflower with an objective to select sufficient antagonists

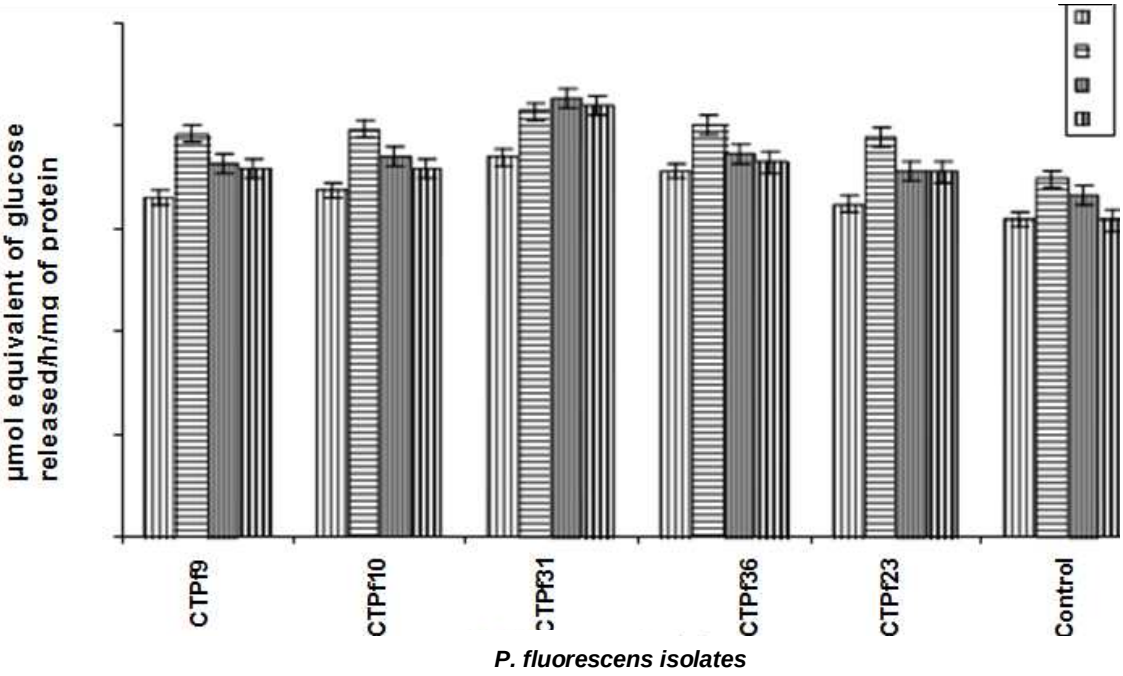

Figure 3. Induction of β 1,3-glucanase activity in safflower treated with *P. fluorescens* isolates against *M. phaseolina*.

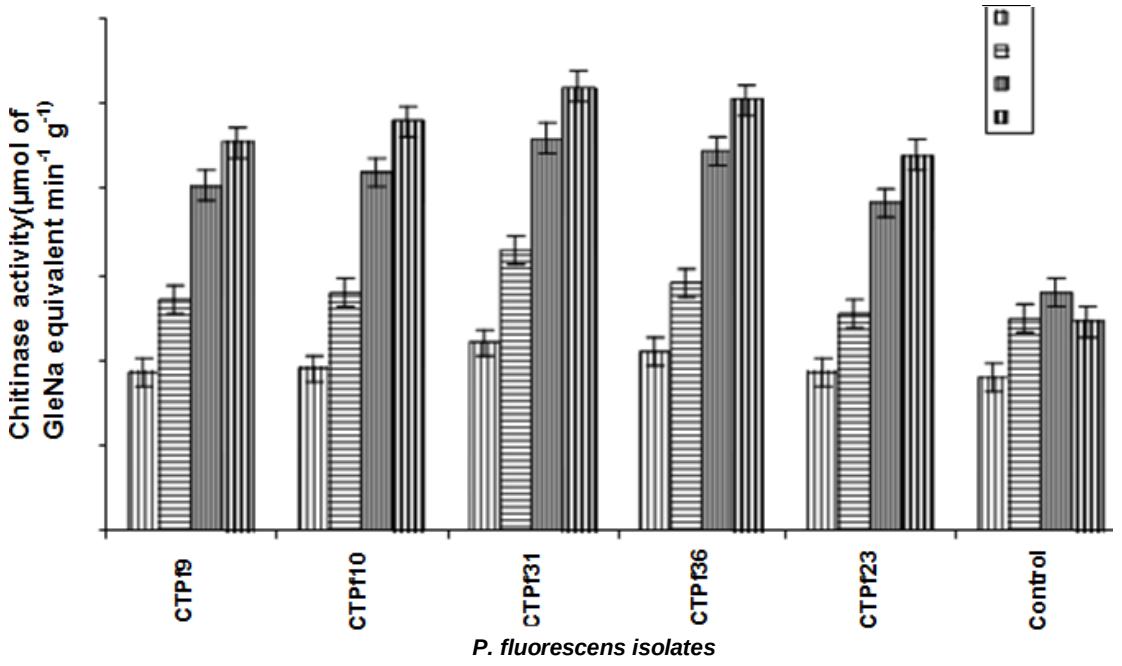

Figure 4. Total chitinase activity in safflower plants treated with *P.fluorescens* isolates after challenge inoculation with *M. phaseolina* at different intervals.

against seed-borne pathogen, *M. phaseolina*. We tried screening methods to select a suitable *P. fluorescens* isolate for the control of safflower root-rot. Dual culture technique on culture media was taken as a measure of antagonistic potential of the isolates from which 13 isolates were found to produce detectable inhibition

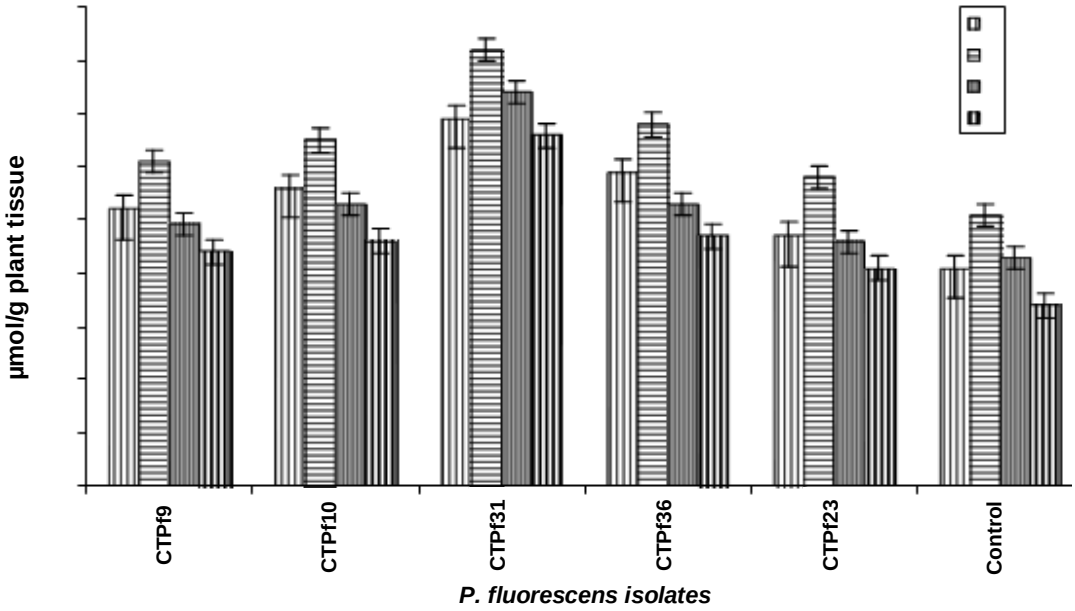

Figure 5. Induction of phenol content in safflower treated with *P. fluorescens* isolates against *M.phaseolina*.

zones against target pathogen. All the 13 isolates were used for seed treatment to test their efficacy in inhibition of the target pathogen and to know their importance in seed germination, seedling growth and seedling vigour of safflower, which better resembled the greenhouse conditions. In greenhouse condition, the host plant plays an important role in supporting the introduced antagonistic bacteria, *P. fluorescens*. Hence a screening system involving the host plant, pathogen and the antagonist is expected to give more realistic picture than dual culture technique. Due to antagonistic bacteria the disease severity in some treatments (CTPf9, CTPf10, CTPf31, CTPf23 and CTPf36) was either delayed or less than that expected for the control. This effect may be due to the ability of antagonistic isolate to survive for at least a limited period or to suppress the growth of the pathogen during the initial stage. The success in controlling the disease caused by *P. fluorescens* bacteria is achieved by their ability to be effective in the early steps of the disease establishment (Guetsky et al., 2001; Yoshida et al., 2001). In standard blotter method, majority of the antagonistic bacteria isolates (CTPf9, CTPf10, CTPf31, CTPf23 and CTPf36) were effective in reducing the percentage incidence of root-rot pathogen. In addition, all of these isolates enhanced the seed germination, root-shoot lengths and seedling vigour compared to other treatments and control. Increased plant growth was observed in the safflower due to *P. fluorescens* isolates and it is mainly dependent on the ability of the organisms to survive and develop in the rhizosphere. Antagonistic bacteria isolated from the rhizosphere of a specific crop may be better adapted to that crop and may provide better control of diseases than organisms originally isolated from other crop (Cook, 1993). In this study, the antagonists which were isolated from the safflower rhizosphere are believed to have better chances of survival and colonization on safflower root than those antagonists isolated from any other crop. In turn *Pseudomonas* spp. play important roles in the biological suppression of the many diseases (Dowling and O'gara, 1992; Whipps, 1999; Rajkumar et al., 2005).

In greenhouse studies for biological control of *M. phaseolina*, isolates were selected based on pattern of inhibition exhibited in the dual culture assay and seed germination as well as seedling vigour in paper towel method. Isolate CTPf9, CTPf10, CTPf16, CTPf19, CTPf21, CTPF31, CTPf34 and CTPf36 showed high inhibition of fungal growth on dual culture in addition to poor suppression of disease incidence and seedling vigour. Isolate CTPf31 was moderate in dual culture and maximum suppression of disease severity in greenhouse conditions. While isolate CTPf9, CTPf10 and CTPf23 showed high inhibition of fungal growth in dual culture technique and they were not able to reduce the disease development in greenhouse conditions. Our results are confirmatory with the findings of many workers (Schroth and Hancock, 1981; Wong and Baker, 1984; Rajkumar et al., 2005). Isolate CTPf31 showed greater reduction of disease severity in greenhouse experiments in addition to increasing the safflower growth. Among the antagonists tested, isolate CTPf31 had the most beneficial characteristics, as it consistently suppressed the root-rot in safflower and also increased the plant growth. The promising results from all the isolates (CTPf9, CTPf10,

CTPf31, CTPf23 and CTPf36) of *Pseudomonas* bacteria given in *in vitro* and greenhouse tests were subjected to taxonomic characterization by Gram staining, biochemical tests and test for utilization of sugars. These tests confirmed the presence of *Pseudomonas fluorescens*.

In this study, fluorescent pseudomonads were preliminarily selected for biological control properties before plant growth stimulation. It is evident from the results that the treatment of antagonistic isolates (CTPf31 and CTPF36) resulted in varying degrees of plant growth. The plant growth promotion by rhizosphere bacteria might be associated with secretion of auxins, gibberllins, cytokinins and indole acetic acid (Dubeikovsky et al., 1993; Ramamoorthy and Samiyappan, 2001), the improvement of nutrients and water uptake (Pleban et al., 1995; Nowak and Lazorovits, 1997) and suppression of deleterious micro-organisms in the rhizosphere (Gamliel and Katan, 1993). The use of rhizosphere bacteria for increasing the yield and crop protection is an attractive approach in the modern system of sustainable agriculture. Peroxidase (PO) activities were increased maximum on the 6th day after challenge inoculation with the target pathogen. The PO catalysed the last step in the biosynthesis of lignin and other oxidative phenols. Zdor and Anderson (1992) noticed that rhizosphere colonization of various bacteria induced PO activity in bean. The higher PO activity was observed in cucumber roots treated with *Pseudomonas corrugate* challenged with *Pseudomonas aphadermatum* (Chen et al., 2000) and seedlings treated with *Pseudomonas* spp. challenged with similar pathogen in chilli (Kavitha et al., 2005). Seed bacterization with plant growth-promoting rhizobacteria (PGPR) showed an increased in PO on the 6th day after challenge inoculation. The results obtained are in agreement with the results of Podile and Laxmi (1998) and Silva et al., (2004). The high peroxidase activities are usually associated with later stages of the infection process and are linked to generation of hydrogen peroxides that inhibit pathogens directly or generate other free radicles with antimicrobial effects (Hammerschmidt et al., 1982). β-1,3-Glucanase activity was increased in PGPR treated safflower plants after 6 days of challenge inoculation with target pathogen. Kavitha et al. (2005) and Benhamou et al. (1996) have reported that seed treatment with PGPR is able to produce hydrolytic enzymes such as chitinases and β-1,3-glucanases in chilli and pea. These host lytic enzymes accumulates at the site of penetration of the fungus *F. oxysporum* f.sp. *pisi, resulting* in the degradation of the fungal cell wall (Benhamou et al., 1996). In a number of plant species, β-1,3-glucanase exists in multiple forms. These enzymes solubilize elicitor's active glucan molecules from the fungal cell wall (Mauch and Staechelin, 1989) and also induce defense enzymes. When the pathogen grows initially in the intercellular spaces, the fungus may come in contact with β-1,3-glucanase localized in the middle lamellae. PAL activity was increased at day 6 after challenge inoculation with target pathogen due to PGPR isolates. Isolate CTPf31, CTPf36 and CTPf10 effectively enhanced the enzyme activity in presence of the pathogen. Similar results were noticed by Meena et al. (2000) in groundnut, Silva et al. (2004) in tomato plant against five pathogens and Kavitha et al. (2005) in chilli against *P. aphadermatum*. The product of PAL cinammic acid, is directly linked to cell lignification processes and the highest levels of PAL activity usually about 24 h after initial infection (Podile and Laxmi, 1998). So, PAL generally shows increased activity during pathogen establishment in the host tissue (Bhattacharya and Ward, 1988). PAL plays an important role in the biosynthesis of phenolic phytoalexins (Daayf et al., 1997). The increase in PAL activity indicates the activation of phenyl propanoid pathway. In several host-pathogen interactions, increased levels of phenyl propanoid have been shown to correlate with incompatibility (Rathmell, 1973; Bhattacharya and Ward 1988; Ralton et al., 1989). The product of PAL is tras cinnamic acid that is an immediate precursor for the biosynthesis of salicylic acid (SA), a signal molecule in systemic acquired resistance (SAR) (Klessig and Malamy, 1994). Increment of phenol was high in seed treatment with CTPf31, CTPf36 and CTPf10 resulted in increased accumulation of phenolic substances in response to infection by the pathogen. M'Piga et al. (1997) reported that *P. fluorescence* induced the accumulation of phenolic substances exhibited considerable morphological changes including cytoplasmic disorganization and loss of protoplasmic content. Ramamoorthy and Samiyappan (2001) reported the accumulation of phenolic substances and pathogen related (PR) proteins in response to infection by *F. oxysporum* f. sp. *lycopersici* in tomato. The results obtained in this study are in agreement with these findings.

Seed treatment with *P. fluorescens* enhanced the plant growth and defense enzymes and decreased the percent incidence of the *M. phaseolina* root-rot. Application of PGPR results in biochemical or physiological changes in safflower plants. Normally, induced systemic resistance (ISR) by PGPR is associated with the accumulation of PR proteins (pathogenesis-related proteins) (M'Piga et al., 1997), synthesis of phytoalexin and other secondary metabolites (Zdor and Anderson, 1992). This study demonstrates that different PGPR isolates and their role in exhibiting adverse effects on seedling wilt of safflower. Moreover, it is concluded that CTPf31, CTPf36 and CTPf10 increased the plant resistance to root-rot pathogen. It is easily imagined that the different isolates of *P. fluorescens* produced antimicrobial products and defense enzymes restrict the development of challenging phytopathogenic fungi.

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Abbreviations: ISR, Induced systemic resistance; **PAL**, phenylalanine ammonia-lyase; **KBM,** King's B medium; **DMRT,** Duncan's new multiple range test; **PO,** peroxidase; **PGPR**, plant growth-promoting rhizobacteria; **SA,** salicylic acid; **SAR** ,systemic acquired resistance; **PR,** pathogenesis related; **ISR,** induced systemic resistance; **PDA,** potato dextrose; **ISTA,** international seed testing association.

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