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Biological Control of Leaf Blight Disease in *Gloriosa superba* using *Pseudomonas fluorescens*

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Leaf blight caused by *Alternaria alternata* is a destructive disease in *Gloriosa superba*. The effect of *Pseudomonas fluorescens* formulation in managing the leaf blight disease and in inducing resistance in gloriosa was investigated. *P. fluorescens* strain Pf1 significantly inhibited the growth of *A. alternata in vitro*. Dipping the tubers followed by foliar application of *P. fluorescens* at the rate of 2 g per litre on 30 and 60 days after planting was effective in managing the leaf blight disease and increasing the seed yield under field conditions. Changes in the activities of phenylalanine ammonia-lyase, chitinase, β-1,3-glucanase and in phenolic content of gloriosa leaves after application of *P. fluorescens* and inoculation with *A. alternata* were measured. There was a marked increase in the induction of defense compounds in response to pathogen inoculation in *P. fluorescens* treated gloriosa leaves.

Key words: Leaf blight, *Gloriosa superba*, *Pseudomonas fluorescens*, defense compounds.

Gloriosa superba L. (Liliaceae) is a tuberous climbing plant with brilliant wavy-edged yellow and red petalled flowers. The name Gloriosa comes from the word gloriosus, which means handsome and superba from the word superb clearly alluding to the beautiful flowers which appear from November to March. Glory lilies are grown commercially for a chemical compound, colchicine. Seeds and tubers contain valuable alkaloids *viz.*, colchicine and colchicoside and used to treat gout and rheumatism. *Gloriosa* seeds contain high level of colchicine as compared to tubers. Cornigerine, 3-demethyl-N-formyl-N-deacetyl-b-lumicolchicine, 3-demethyl-g-lumicolchicine, 3-demethyl colchicines have been isolated from plant. It is used as anthemirtic, alexiteric and useful in ulcers, piles, abdominal pains and itching.

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Leaf blight disease caused by the fungus, *Alternaria alternata* (Fr.) Keissler is the destructive disease in *G superba*. Smyptoms appear in all the stages of plant growth. Initially, small, pale to brown, irregular or round spots appear on the leaves. Each spot has a central necrotic lesion with concentric rings. In advanced stages, several spots coalesce together to form large blighted areas. The disease also occurs on the petiole and flowers. The pollination gets affected due to leaf blight infection and consequently the yield is drastically reduced. The loss due to leaf blight infection in *G. superba* was estimated to be 65 to 80% (Maiti *et al.,* 2007).

Spores of *Alternaria* sp. are dark brown to black and appear in felty black masses on leaves. They generally move by water splashing or air movement. Management of disease through fungicides alone leads to cause soil residual problem and health hazards, besides involving higher input cost. The use of microbial agents can be an attractive option for control of plant diseases.

Fluorescent pseudomonads have received particular attention throughout the globe because of their catabolic versatility, excellent root-colonizing abilities and their capacity to produce a wide range of antifungal metabolites. Biopesticides are cheaper, ecofriendly and do not pose risk of the pathogen developing resistance. Hence, the present research is carried out to manage leaf blight disease of *Gloriosa superba* using *Pseudomonas fluorescens*.

MATERIALS AND METHODS

Isolation and *in vitro* screening of *Pseudomonas fluorescens* against *Alternaria alternata*

The pathogen *A. alternata* was isolated from the infected leaves of *G. superba* and maintained on potato dextrose agar (PDA) slants. Using King's B medium, fluorescent pseudomonads were isolated from the rhizosphere of various crop plants (King *et al.*, 1954). The strains were identified as fluorescent pseudomonads according to Bergey's Manual of Systematic Bacteriology. The antagonistic potential of biocontrol agents against *A. alternata* was tested by dual culture method on PDA medium (Dennis and Webster, 1971). A 9 mm actively growing culture disc of pathogen and antagonists were separately placed or streaked opposite to each other on sterilized PDA medium under aseptic conditions. The medium inoculated with pathogen alone served as the control. The radial growth of the pathogen was measured after seven days of incubation. Four replications were maintained.

Field studies

A talc-based powder formulation of *P. fluorescens* was developed as described by Vidhyasekaran and Muthamilan (1995). Field eperiments were conducted in the farmers field at Vellipalayam, Coimbatore District, Tamil Nadu on the management of leaf blight disease of *Gloriosa superba* during 2012-2013 and 2013-2014. The tubers of gloriosa were dipped in *P. fluorescens* formulation at the rate of 2 g per litre for 20 minutes. Foliar application of *P. fluorescens* formulation was done on 30 and 60 days after planting. The combination treatment of dipping the tubers and foliar applicaton with *P. fluorescens* was also made. For comparison, the tubers were dipped in fungicide carbendazim at the rate of 2 g per litre for 20 minutes and spraying with carbendazim at 2 g per litre was done on 30 and 60 days after planting. The leaf blight disease intensity was assessed on 90 days after planting using 0-9 disease rating scale as described by Pawelec *et al*., 2006 [0 : No visible disease damage; 1 : <5% leaf area damaged; 3 : 5-20% leaf area damaged; 5 : 20-40% leaf area damaged; 7 : 40-60% leaf area damaged; 9 : severe defoliation].

Per cent disease index (PDI) was calculated using the formula

$$PDI = \frac{\text{Sum of numerical ratings}}{\text{Number of leaves examined x Maximum grade available in the score chart}} \times 100$$

The growth parameters *viz.*, plant height, number of primary branches, number of secondary branches and number of leaves per plant were recorded on 60 DAP. The yield parameters *viz*., number of flowers per plant, number of pods per plant, number of seeds per pod and tuber yield per plant were also observed. The seed yield per hectare was recorded for each treatment and the data were statistically analyzed.

Induction of defense compounds in *G. superba* due to foliar application of *P. fluorescens*

Plants were sprayed with *P. fluorescens* at 30 days after planting and challenge inoculated with *A. alternata*. At various times after application, leaf samples were collected and various analyses were made.

Estimation of phenylalanine ammonia lyase activity

One g of sample was homogenized in 2 ml of ice cold 0.1 M sodium borate buffer, pH 7.0 and centrifuged at 10,000 rpm for 20 min at 4ºC. The supernatant was used to assay the enzyme activity. PAL activity was determined as the rate of conversion of L-phenylalanine to transcinnamic acid at 290 nm (Dickerson *et al*., 1984). Sample extract of 0.4 ml was incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 1 ml of 12 mM L-phenylalanine and incubated for 1 h at 30ºC. The reaction was initiated by the addition of L-phenylalanine and stopped with 0.5 ml of 2 N HCl. A blank was maintained by adding L-phenylalanine after the addition of 2 N HCl. The absorbance was read at 290 nm and the results were expressed as nmol transcinnamic acid min^{-1} g^{-1} of fresh tissue.

Estimation of phenolic content

Leaf samples were homogenized in 10 ml of 80% methonal and agitated for 15 min at 70ºC. To 1 ml of the extract, five ml of distilled water and

250 µl of Folin-ciocalteau reagent (1 N) were added and incubated at 25ºC for 3 min. After that 1 ml of 20% sodium carbonate was added and mixed well. Then the tubes were placed in boiling water for 1 min and cooled. The absorbance was read at 750 nm and catechol was used as the standard. The total phenol content was expressed as µg of catechol g^{-1} of fresh tissue (Zieslin and Ben-Zaken, 1993).

Assay of chitinase

The colorimetric assay of chitinase was carried out as per Boller and Mauch (1988). One gram of sample was extracted with 5 ml of 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was used as enzyme source. The reaction mixture consisted of 10 µl of 0.1 M sodium acetate buffer (pH 4.0), 0.4 ml enzyme source and 0.1 ml colloidal chitin (10 mg). After incubation for 2 h at 37ºC, the reaction was stopped by centrifugation at 3,000 rpm for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl of 1 M potassium phosphate buffer (pH 7.0) and incubated with 20 µl of 3% (w/v) snail gut enzyme for 1 h. Later, the reaction mixture was brought to pH 8.9 by the addition of 70 µl 0.1 M sodium borate buffer (pH 9.8). The mixture was incubated in a boiling water bath for 3 min and rapidly cooled. After addition of 2 ml of para-dimethyl aminobenzaldehyde (DMAB), the mixture was incubated for 20 min at 37°C and the absorbance was measured at 585 nm. N-acetylglucosamine (GlcNAc) was used as a standard and the enzyme activity was expressed as nmol GlcNAc equivalents min^{-1} mg^{-1} of fresh tissue.

Assay of β-1,3-glucanase

One gram of sample was extracted in 5 ml of 0.05 M sodium acetate buffer (pH 5.0). The homogenate was centrifuged at 10,000 g for 10 min. at 4°C and the supernatant was used as enzyme source. β-1,3-glucanase activity was assayed by the laminarin-dinitrosalicylic acid method (Pan *et al.*, 1991). The reaction mixture consisted of 62.5 µl of 4% laminarin and 62.5 µl of enzyme source. The reaction was carried out at 40°C for 10 min and stopped by adding 375 µl of dinitrosalicylic acid and heating for 5 min in boiling water. The absorbance was measured at 500 nm and the enzyme activity was expressed as nmol GlcNAc equivalents min^{-1} mg^{-1} of fresh tissue.

RESULTS AND DISCUSSION

In vitro studies revealed that among the various strains of *P. fluorescens* tested against the growth of *A. alternata*, Pf1 strain was the most effective in inhibiting the pathogen by recording least mycelial growth of 2.8 cm. The strain Pf4 was found to be the least effective in inhibiting the growth of *A. alternata* (6.8 cm). The maximum mycelial growth of 8.8 cm was observed in the control (Table 1). The inhibition zone was found to be maximum of 18.2 mm in Pf1 strain and the least inhibition zone (8.1 mm) was recorded in Pf4 strain (Table 1). Meena and Marimuthu (2012) reported that *P. fluorescens* inhibited the growth of *Sclerotium rolfsii in vitro* effectively. The complete inhibition of the growth of *Ganoderma lucidum*, basal stem rot pathogen by the volatile metabolites of *P. fluorescens* was highlighted by Sreenivasulu *et al*. (2006).

The results of the field experiments revealed that dipping the tubers in *P. fluorescens* (2 g/l) for 20 minutes followed by spraying with *P. fluorescens* (2 g/l) twice on 30 and 60 days after planting was effective in managing the leaf blight disease which recorded the lowest disease intensity of 19.7 per cent (Table 2). Tuber treatment with *P. fluorescens* alone was effective *in* managing leaf blight disease of *G. superba*. However, additional foliar application of *P. fluorescens* formulation increased its efficacy. The efficacy was comparable with that of carbendazim treatment. The

Table 1. *In vitro* inhibition of *Alternaria alternata* by *Pseudomonas fluorescens*

P. fluorescens strains	Mycelial growth of *A. alternata**(cm)	Inhibition zone(mm)
Pf1	2.8	18.2
Pf2	5.9	8.6
Pf3	4.1	13.4
Pf4	6.8	8.1
Pf5	4.6	11.3
Control	8.8	-
CD (p=0.05)	1.4	

* Mean of four replications

highest leaf blight disease intensity of 34.6 per cent was observed in the control (Table 2). The effectiveness of *Pseudomonas fluorescens* in the management of foliar diseases had been reported in several crops (Meena *et al.,* 2002; Sreenivasulu *et al.,* 2006; Harish *et al*., 2008; Meena and Marimuthu, 2012).

The growth parameters *viz.,* plant height (120.4 cm), number of primary branches (2.6), number of secondary branches (4.8) and number of leaves per plant (110.4) were found to be the maximum in dipping the tubers and spraying with *P. fluorescens* (2 g/l) (Table 3). In control, the plant growth parameters *viz.*, plant height (96.1 cm), number of primary branches (1.8), number of secondary branches (3.2) and number of leaves per plant (80.2) were found to be the minimum (Table 3). *P. fluorescens* has the ability to produce plant growth hormones and or antimicrobial substances and to protect growing roots from deleterious root microbes present in the rhizosphere (Harish *et al.,* 2008). Treating with *P. fluorescens* recorded the maximum yield parameters *viz.*, number of flowers per plant (56.4), number of pods per plant (47.1), number of seeds per pod (74.6) and tuber yield per plant (88.4 g). The yield parameters were found to be on par with carbendazim treatment (Table 4). In the control, yield parameters *viz.*, number of flowers per plant (35.4), number of pods per plant (24.2), number of seeds per pod (54.2) and tuber yield per plant (61.3 g) were found to be the lowest (Table 4). The maximum seed yield of 520.2 kg/ha was recorded in dipping the tubers and spraying *P. fluorescens* at 2 g per litre. In the control, minimum seed yield of 341.4 kg/ha was recorded (Table 2). Several

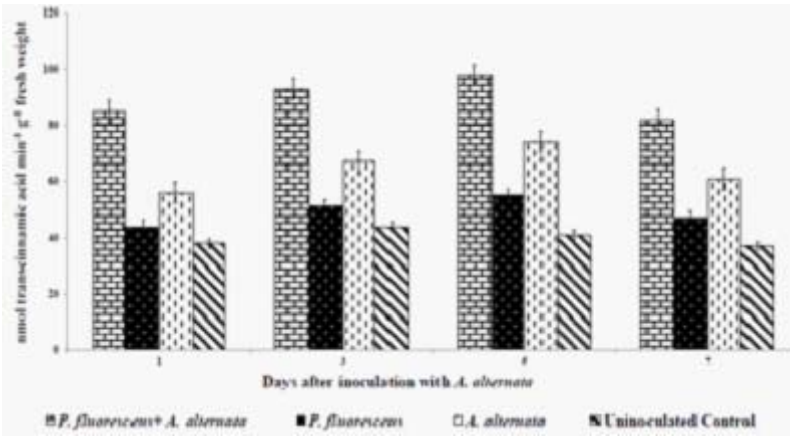

Fig. 1. Effect of *P. fluorescens* on PAL activity in *G. superba* leaves

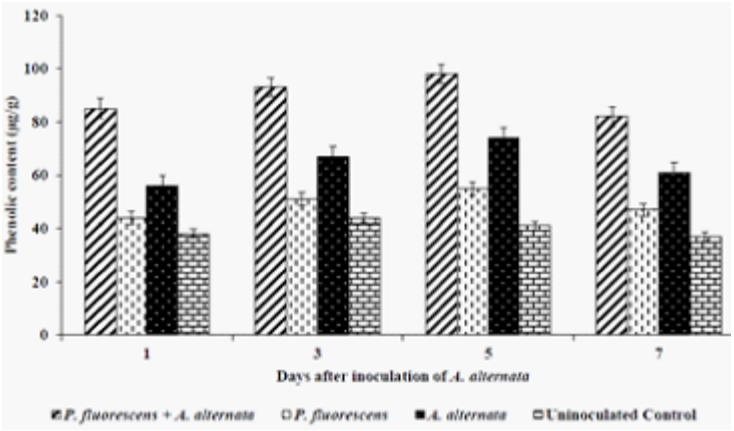

Fig. 2 . Effect of foliar application of *P. fluorescens* on accummulation of phenols in *G. superba*

fluorescent Pseudomonads were known to control fungal pathogens like *Pythium, Fusarium, Rhizoctonia* in a wide range of crops (Vidhyasekaran *et al.*, 1997).

In the present study, it was observed that foliar application of *P. fluorescens* induced the accumulation of phenolics and increased the activity of PAL in *G. superba* leaves. The highest PAL activity was observed when *G. superba* leaves were treated with *P. fluorescens* and challenge inoculated with *A. alternata*. The enzyme activity reached the maximum on fifth day of challenge inoculation and thereafter it declined (Fig. 1). The application of *P. fluorescens* in gloriosa leaves and challenged with *A. alternata* resulted in increased accumulation of phenolics than the individual treatment (Fig. 2). There was a significant increase in phenol activity against the control due to the colonization by growth promoting bacteria. Treatment combinations of *P. fluorescens* on gloriosa leaves and challenged with *A. alternata* play a major role in inducing phenols in gloriosa leaves than the individual treatment (Fig. 2). PAL is the first enzyme of phenylpropanoid metabolism in higher plants and it has been suggested to play a significant role in regulating the accumulation of phenolics. Since the production of phenolic compounds depends upon PAL activity (Graham and Graham, 1991), increased phenolic synthesis in *P. fluorescens* treated leaves may be due to increased activity of PAL.

In the present study, it was also observed that application of *P. fluorescens* as foliar application induced the accumulation of lytic enzymes in *G. superba.* Assay of chitinase from the bioagent treated leaves challenged with leaf

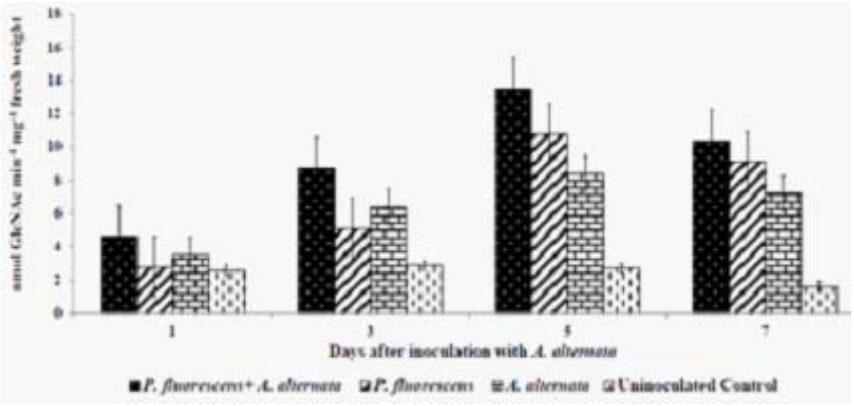

Fig. 3. Effect of *P. fluorescens* formulation on ehitinase activity in leaves of *G. superba*

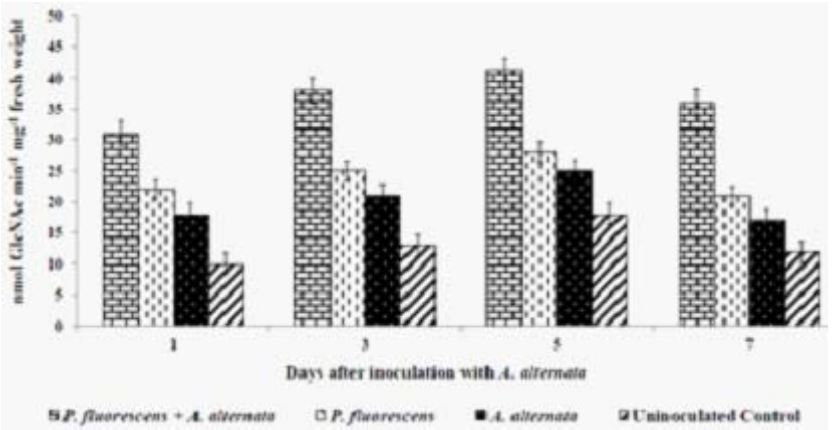

Fig. 4. Effect of foliar application of *P. fluorescens* on β,1-3 glucanase activity in leaves of *G. superba*

blight pathogen showed the enhanced activity rather than the untreated control leaves. The effect of *P. fluorescens* on chitinase activity in gloriosa leaves with or without *A. alternata* was observed. Among the different treatments, treating gloriosa leaves with *P. fluorescens* and challenged with *A. alternata* recorded higher activity of chitinase than the uninoculated control leaves (Fig 3). The results in Fig 4 showed that the activity of β-1,3-glucanase was maximum in *P. fluorescens* treated leaves and challenged with *A. alternata* than uninoculated control leaves. In gloriosa leaves inoculated with *A. alternata*, the enzyme activity increased but not to the level of bioagent treated leaves (Fig 4). Several microbial strains protect plants from various pests and diseases by activating defense genes encoding chitinase, glucanase, peroxidase, phytoalexin synthesis and

Table 2. Effect of *Pseudomonas fluorescens* formulation in the management of leaf blight disease of *Gloriosa superba* (Pooled mean of Trial I and Trial II)

Treatments	Leaf blight disease intensity PDI	Disease reduction over control (%)	Seed yield (kg/ha)	Increased yield over control (%)
T_1- Dipping the tubers in *Pseudomonas fluorescens* @ 2 g/l for 20 minutes	24.1 (29.1)	30.3	456.1	25.1
T_2- Foliar spraying of *P. fluorescens* @ 2 g/l on 30 DAP & 60 DAP	22.6 (27.3)	34.7	412.4	17.2
T_3- Dipping the tubers + Foliar spraying of *P. fluorescens* @ 2 g/l on 30 DAP & 60 DAP	19.7 (15.6)	43.1	520.2	34.4
T_4- Dipping the tubers in carbendazim 0.1% for 20 minutes	24.8 (29.8)	28.3	434.4	21.4
T_5- Foliar spraying of carbendazim 0.1% on 30 DAP and 60 DAP	21.4 (27.2)	38.2	416.6	18.1
T_6- Dipping the tubers + Foliar spraying of carbendazim 0.1% on 30 DAP and 60 DAP	19.1 (15.4)	44.8	512.6	33.4
T_7- Control	34.6 (352)	-	341.4	-
CD (P=0.05)	1.8		34.3	

Mean of three replications; Figures in the parentheses are arcsine transformed values

Table 3. Effect of fungicides and biocontrol agents on plant growth parameters of *G. superba*

Treatments	Plant height (cm)	No. of primary branches	No. of secondary branches	No. of leaves per plant
T_1-Dipping the tubers in *Pseudomonas fluorescens* @ 2 g/l for 20 minutes	118.3	2.3	4.1	112.3
T_2-Foliar spraying of *P. fluorescens* @ 2 g/l on 30 DAP & 60 DAP	114.2	2.3	3.6	106.1
T_3-Dipping the tubers + Foliar spraying of *P. fluorescens* @ 2 g/l on 30 DAP & 60 DAP	120.4	2.6	4.8	110.4
T_4-Dipping the tubers in carbendazim 0.1% for 20 minutes	105.6	2.3	3.8	97.8
T_5-Foliar spraying of carbendazim 0.1% on 30 DAP and 60 DAP	103.4	1.8	3.4	96.4
T_6-Dipping the tubers + Foliar spraying of carbendazim 0.1% on 30 DAP and 60 DAP	110.8	2.6	4.1	114.5
T_7- Control	96.1	1.8	3.2	80.2
CD (P=0.05)	3.6	0.4	0.6	4.3

Mean of three replications

Table 4. Effect of treatments on yield parameters of *Gloriosa superba*

Treatments	No. of flowers per plant	No. of pods per plant	No. of seeds per pod	Fresh tuber yield per plant (g)
T_1- Dipping the tubers in *Pseudomonas fluorescens* @ 2 g/l for 20 minutes	51.2	43.4	70.4	80.1
T_2-Foliar spraying of *P. fluorescens* @ 2 g/l on 30 DAP & 60 DAP	52.6	42.3	66.1	74.2
T_3- Dipping the tubers + Foliar spraying of *P. fluorescens* @ 2 g/l on 30 DAP & 60 DAP	56.4	47.1	74.6	88.4
T_4- Dipping the tubers in carbendazim 0.1% for 20 minutes	42.1	33.6	66.2	85.3
T_5- Foliar spraying of carbendazim 0.1% on 30 DAP and 60 DAP	45.3	34.2	65.3	86.4
T_6- Dipping the tubers + Foliar spraying of carbendazim 0.1% on 30 DAP and 60 DAP	50.7	41.5	68.4	90.6
T_7- Control	35.4	24.2	54.2	61.3
CD (P=0.05)	2.6	2.8	2.1	2.4

Mean of three replications

inducing physiological changes (Rajendran *et al.,* 2007; Saravanakumar *et al.,* 2007; Harish *et al.*, 2008). The increased activities of PAL, lytic enzymes and accumulation of phenolics in *G. superba* in response to foliar application of *P. fluorescens* might have contributed to increase resistance against *A. alternata* in the present study.

The present study indicated the usefulness of *P. fluorescens* formulation for the management of leaf blight disease in *G. superba*. Studies conducted so far have thus reinforced the prospects of using *P. fluorescens* on a commercial scale as a successful alternative for chemical control of foliar diseases.

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REFERENCES

1. Boller, T., Mauch, F. Colorimetric assay for chitinase. *Meth. Enzymol*., 1988; **161**: 430-435.
2. Dennis, C., Webster, J. Antagonistic properties of species groups of *Trichoderma :* Production of non-volatile antibiotics. *Trans. Br. Mycol. Soc.,* 1971; **57**: 25-39.
3. Dickerson, D.P., Pascholati, S.F., Hagerman, A.E., Butler, L.G Nicholson, R.L. Phenylalanine ammonia-lyase and hydroxycinnamate : CoA ligase in maize mesocotyls inoculated with *Helminthosporium maydis* or *Helminthosporium carbonum*. *Physiol. Pl. Path.,* 1984; **25**: 111-123.
4. Graham, T.L., Graham, M.Y. Cellular coordination of molecular responses in plant defenses. *Mol. Pl. Microbe Interact*., 1991; **4**: 415-422.
5. Harish, S., Kavino, M., Kumar, N., Saravana kumar, D., Soorianathasundaram, K., Samiyappan, R. Biohardening with Plant Growth Promoting Rhizosphere and Endophytic bacteria induces systemic resistance against Banana bunchy top virus. *Applied Soil Ecology*, 2008; **39**: 187–200.
6. King, E.O., Ward, M.K., Raney, D.E. Two simple media for the demonstration of pyocyanine and fluorescein. *J. Lab. Clin. Med*., 1954; **44**: 301-307.
7. Maiti, C.K., Surjit, S., Amal, K.P., Krishnendu, A. First report of leaf blight disease of *Gloriosa superba* L. caused by *Alternaria alternata* (Fr.) Keissler in India. *J. Gen. Plant Pathol*., 2007; **73**: 377-378.
8. Meena, B., Marimuthu, T. Effect of application methods of *Pseudomonas fluorescens* for the late leaf spot of groundnut management. *JBiopest.,* 2012; **5**: 14-17.
9. Meena, B., Marimuthu, T. Exploitation of biocontrol agents for the management of jasmine wilt. *South Indian Hort.,* 2012; **60**: 187-189.

10. Meena, B., Radhajeyalakshmi, R., Marimuthu, T., Vidhyasekaran, P., Velazhahan, R. Biological control of groundnut late leaf spot and rust by seed and foliar applications of a powder formulation of *Pseudomonas fluorescens*. *Biocontrol Sci Technol.,* 2002; **12**: 195-204.
11. Pan, S.Q., Ye, X.S., Kuc, J. Association of β-1,3-glucanase activity and isoform pattern with systemic resistance to blue mould in tobacco induced by stem injection with *Peronospora tabacina* or leaf inoculation with tobacco mosaic virus. *Physiol. Mol. Plant Patho*l., 1991; **39**: 25-39.
12. Rajendran, L., Samiyappan, R., Raguchander, T., Saravanakumar, D. Endophytic bacteria mediate plant resistance against cotton bollworm. *Journal of Plant Interactions*, 2007; **2:** 1-10.
13. Saravanakumar, D., Vijayakumar, C., Kumar, N., Samiyappan, R. PGPR – induced defense responses in the tea plant against blister blight disease. *Crop Protection*, 2007; **26**: 556-565.
14. Sreenivasulu, B., Krishnakumar, K.V., Aruna, K., Lakshmi, M.V., Rao, D.V.R. Biointensive IDM approach against basal stem rot and stem bleeding disease of coconut. *Journal of Plantation Crops*, 2006; **34**: 502–507.
15. Vidhyasekaran, P., Muthamilan, M. Development of formulations of *Pseudomonas fluorescens* for control of chickpea wilt. *Pl. Dis*., 1995; **79**: 782-786.
16. Vidhyasekaran, P., Sethuraman, K., Rajappan, K., Vasumathi, K. Powder formulations of *Pseudomonas fluorescens* to control pigeonpea wilt. *Biological Control*, 1997; **8**: 166-171.
17. Zieslin, N., Ben-Zaken, R. Peroxidase activity and presence of phenolic substances in peduncles of rose flowers. *Plant Physiol. Biochem*., 1993; **31**: 333-339.