Research Paper :

Induced Systemic Resistance by *Methylobacterium extorquens* against *Rhizoctonia solani* in Cotton

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SUMMARY

The use of microorganisms for biological purposes has become an effective alternative to control plant pathogens. There are many examples of formulations using bacterial or fungal strains with biocontrol applications. Among them, members of the genus *Methylobacterium* are well-known growth regulator producers and also having *in vitro* biocontrol ability of against the phytopathogen, *Rhizoctonia solani.* Four Methylobacterial isolates CO-47, MV-10, AM1 and LE-1 were selected for assessing their *in vitro* biocontrol activity. Among the various isolates of *Methylobacterium* sp. screened, the isolate CO 47 significantly reduced the linear mycelial growth of *Rhizoctonia solani* to an extent of 52.2 per cent over control with an inhibition zone of 1.4 cm under *in vitro* conditions. Based on the result *in vitro* conditions CO-47 caused the maximum inhibition of *R. solani.* Under pot culture conditions, soil application with *Methylobacterium extorquens* CO-47 challenge inoculated with *R. solani* induced accumulation of peroxidase, polyphenoloxidase, phenylalanine lyase and phenols resulting in suppression of *R. solani.*

Key words : *Methylobacterium extorquens*, cotton, PR proteins, *R. solani*

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Biological control is an enviroment friendly strategy to reduce crop damage caused by plant pathogens. Biological control of soil borne pathogens with antagonistic bacteria and fungi has been intensively investigated. Fluorescent Pseudomonads have revolutionized the field of biological control of soil borne plant pathogens. During the last 25 years, they have emerged as the largest potentially most promising group of plant growth promoting Rhizobacteria involved in the biocontrol of plant disease. In this view, a new bacterium pink pigmented facultative methylotrophs (PPFMs) having biocontrol activity. Pink pigmented facultative methylotrophs belonging to the genus *Methylobacterium*, are a physiologically interesting group of bacteria that preferentially utilize methanol and other reduced one carbon compounds such as formate and formaldehyde as sole source of carbon and energy *via* serine pathway (Green, 1992). Members of the genus *Methylobacterium* are ubiquitous in nature and are thus found in a variety of habitats (Green and Bousifield, 1981, 1983) including soil, dust, fresh water, lake sediments, leaf surfaces, nodules and rice grains.The beneficial effect of *Methylobacterium* in plants interms of seed germination, seedling establishment and plant productivity and also induce pathogenesis related proteins has been main focus in the recent past. Attack of pathogens due to production of diverse microbial metabolites like siderophore and plant growth enhancement IAA and cytokinin production.Biocontrol research has gained considerable attention and appears promising as a viable alternative to chemical control strategies. *Methylobacterium* can induce physiological changes throught out entire plants, making them more resistant to pathogens.This phenomenon, termed induced systemic resistance (ISR), has been demonstrated for various rhizobacteria in several plants.The induced systemic resistance reduces disease symptoms of wide range of pathogens and its physiological characterization is in progress.In some cases, ISR by rhizobacteria are characterized by systemic accumulation of pathogenesis related proteins that is also associated with an accumulation of pathogenesis related proteins.

MATERIALS AND METHODS

Bacterial, fungal and cotton seed collections:

Methylobacterium sp. strain Co-47, MV-10, LE-1 AM-1 and *Rhizoctonia solani* obtained from the Department of Agricultural Microbiology and plant pathogen were obtained from the Department of Plant pathology, Agricultural College and Research Institute,

Coimbatore, Tamil Nadu.

Culture maintaence:

Methylobacteria were grown for 72 h on Ammonium mineral salt (AMS) medium (pH 6.8) supplemented with methanol 0.5% and cycloheximide (30µg ml^{-1}). All the fungal pathogens were maintained and grown on Potato dextrose agar(PDA) before use.

Antagonistic effect of Methylobacterium against Rhizoctonia solani:

In vitro screening of antagonistic bacteria:

The antifungal efficacies of *Methylobacterium* strains were tested by dual culture technique (Dennis and Webster, 1971) using PDA medium. A mycelial disc of the pathogen (5mm dia.), *R.solani* was placed at one end of the Petriplate. The bacterial antagonists were streaked 1 cm away from the periphery of the Petriplate just opposite to the mycelial disc of the pathogen. Visual observation on the inhibition of the growth of fungal pathogen were recorded after 96 hours of incubation in comparison with the PDA plate simultaneously inoculated with only fungal pathogen.

Antagonistic effect of bacteria in liquid PDA broth:

The PDA broth was prepared and four different cultures of *Methylobacterium* were inoculated in 1%. A mycelial disc of the pathogen (5mm dia.), *R.solani* (2 disc) inoculated in same time and incubated for 5-7 days after that mycelial weight was measured.

Per cent inhibition (PI):

Per cent inhibition of test pathogen by the antagonistic strains was evaluated by dual culture technique (Dennis and Webster, 1971). The radial growth of mycelium in mm of antagonist and pathogen was measured and per cent inhibition (PI) was calculated.

$$PI = \frac{C - T}{C} \times 100$$

where, C is the growth of test pathogen (mm) in the absence of the antagonist strain; T is the growth of test pathogen (mm) in the presence of the antagonist strain.

Induction of defense related enzymes against root rot of cotton by Methylobacterium:

A pot culture experiment was carried out with 3 treatments and 3 replications per treatment and details as described in earlier. The cotton seeds were sown in the pots at the rate of 10 seeds per pot and different observations were recorded.

Preparation of pot mixture:

Red soil having the chemical properties of pH 7.4, EC 0.4 dSm^{-1}, available nitrogen 230 kg ha^{-1}, available P_2O_5 10 kg ha^{-1} and available K_2O 250 kg ha^{-1} was passed through a 4mm sieve and mixed along with farmyard manure in 2:1 proportion and filled in pot of 47 x 30 cm size at the rate of 4 kg pot.

Treatment details:

The experiment was designed in RBD with3 treatments and 3 replications. The inorganic fertilizers at 80:40:40 NPK (kg ha^{-1}) were applied to soil as basal dressing.

T_1 – Control

T_2 – Soil application with *R.solani*

T_3 – Soil application with *Methylobacterium extorquens* CO 47 + *R.solani*

Soil application with PPFM cultures:

Standard inoculum of PPFMs (diluted at 1:100 ratio with sterile water) was mixed with lignite till 40 per cent moisture holding capacity and the pH of the lignite was adjusted to neutral with $CaCO_3$. Then the lignite along with the PPFM culture was applied to the soil at the rate of 2 kg ha^{-}

Soil application of fungal pathogens:

The root rot pathogen, *R. solani* was initially grown in PDA medium for 5 days. A mycelial disc of 8 mm size was inoculated into Sand maize medium (19:1 sand: maize). It was incubated at room temperature for 15 days. Pathogenesis was proved by inoculating the pathogen at 2% inoculum levels in the potting mixture.

Effect of Methylobacterium on the induction of defense related enzyme in cotton plants:

Two weeks old cotton plants were treated with the biocontrol agent, *Methylobacterium extorquens* CO 47 by soil application and challenged with the *Rhizctonia solani* pathogen. Leaves were collected from cotton plants on 0, 1, 3, 5, and 7 days after challenge inoculation with *Rhizoctonia solani* and washed several times with sterile distilled water before enzyme extraction. The enzymes were extracted from leaves at ice-cold condition (5° C). The samples were homogenized with phosphate buffer (1 g of leaf with 1 ml of sodium phosphate buffer (0.1M) pH 7.0). The homogenates were centrifuged at 10,000 rpm for 15 min. The supernatant was used as enzyme source for peroxidase (PO), polyphenol oxidase

(PPO), phenylalanine ammonia lyase (PAL) and phenols.

Peroxidase activity (PO):

One gram of fresh leaf sample was ground in 1 ml of 0.1M phosphate buffer pH 7.0 in a pre cooled pestle and mortar. The homogenate was centrifuged at 15,000 g at 4° C for 15 min. The supernatant was used as enzyme source. The reaction mixture consisted of 1.5 ml of 0.05M pyrogallol, 0.1 ml of enzyme extract and 0.5 ml of one per cent H_2O_2. The change in absorbance of the reaction mixture was recorded at 420 nm at 30 sec interval for 3 min at room temperature (28 ± 2° C). The boiled enzyme preparation served as blank. The enzyme activity was expressed as change in absorbance of the reaction mixture per min. per g of leaf sample (Hammerschmidt *et al.*, 1982).

Polyphenol oxidase (PPO):

One gram of fresh leaf sample was ground in 1 ml of 0.1 M sodium phosphate buffer (pH 6.5). The homogenate was centrifuged at 15,000g for 15 min. at 4° C and the supernatant was used as the enzyme source. The reaction mixture consisted of 1.5 ml of 0.1M sodium phosphate buffer pH 6.5 and 0.1 ml of the enzyme extract. The reaction was initiated by the addition of 0.2 ml of catechol (0.01M). The activity was expressed as change in absorbance at 495 nm at 30 sec interval for 3 min. The enzyme activity was expressed as change in absorbance per min. per g of leaf sample (Mayer *et al.*, 1965).

Phenylalanine ammonia lyase (PAL):

Five hundred mg of leaf sample was homogenized in 5 ml of cold 25mM borate HCl buffer (pH 8.8) containing 5mM mercaptoethanol 0.4 ml per l. The homogenate was centrifuged at 15,000g for 15 min. and the supernatant was used as enzyme source. The assay mixture consisted of 0.2 ml of enzyme extract, 1.3 ml water and 0.5 ml borate buffer. The reaction was initiated by the addition of 1 ml of 12mM L-Phenylalanine. The reaction mixture was incubated for 1 h at 32°C. The reaction was stopped by the addition of 0.5 ml of 2N HCl. A blank was run in which phenylalanine was added after adding 2N HCl. The absorbance was measured at 290 nm. The enzyme activity was expressed as mol of cinnamic acid/ min/ g of leaf sample (Dickerson *et al*., 1984).

Phenols:

One gram of the leaf sample was ground in a pestle and mortar in 10 ml of 80 per cent methanol. The homogenate was centrifuged at 10,000 g for 20 min. The supernatant was evaporated to dryness and the residue was dissolved in 5 ml of distilled water. From this, 0.2 ml was taken and the volume was made up to 3 ml with distilled water. To that 0.25 ml of Folin-Ciocalteau reagent (1N) was added. After 3 min., 1 ml of 20 per cent sodium carbonate was added and mixed thoroughly. Then the tubes were placed in boiling water for 1 min. and cooled. The absorbance was measured at 725 nm against a reagent blank. The phenol activity was expressed in mg of catechol per g of leaf sample (Zieslin and Ben Zaken, 1993).

The results of the experiments were subjected to statistical scrutiny as per the usual methods.

RESULTS AND DISCUSSION

The results obtained from the present investigation are summarized below :

Antagonistic potential of methylobacterial isolates against Rhizctonia solani:

The phyllosphere microbial community is an open system. Biocontrol specifically in the phyllosphere has been extensively reviewed since 1980. Blakeman and Fokkema (1982) discussed the potential for biological control of plant diseases in the phylloplane.The various isolates of *Methylobacterium* sp. screened, the isolate CO 47 significantly reduced the linear mycelial growth of *Rhizoctonia solani* to an extent of 52.2 per cent over control with an inhibition zone of 1.4 cm under *in vitro* conditions. In liquid culture CO 47 reduced the mycelial weight of *Rhizoctonia solani* recording 32.5g compared to control recording 65.5g (Table 1 and 2). It was followed by AMI, MV10 and LE1 recorded respective per cent inhibitions of 48.8,46.6 and 33.3 and inhibition zones of 1.2,1.0 and 0.5cm. Fluorescent pseudomonads have revolutionized the field of biological control of soil borne plant pathogens. In the past three decades numerous

Table 1 : *In vitro* screening of antagonistic potential of *Methylobacterium extorquens* strains on mycelial growth of *Rhizoctonia solani* in dual culture method on solid medium

Methylobacterium extorquens strains	Mycelial growth of *R.solani* (mm)	Inhibition zone (cm)	Per cent inhibition of mycelial growth over control
CO 47	43.33	1.4	52.20
AM1	46.00	1.20	48.80
MV10	58.25	1.00	46.60
LE1	62.54	0.5	33.30
Control	90.00	-	

Table 2 : *In vitro* screening of antagonistic potential of *Methylobacterium extorquens* strains in PDA broth on mycelial growth of *Rhizoctonia solani*

Methylobacterium extorquens strains	Mycelial dry weight (g)	Per cent reduction of mycelial growth over control
CO 47	32.50	50.38
AM1	36.20	44.73
MV10	38.25	41.60
LE1	40.50	38.16
Control	65.50	-

strains of fluorescent pseudomonads have been isolated from the soil and plant roots by several workers and their biocontrol activity against soil-borne and foliar pathogens were reported (Cheng *et al.*, 1993). However, the research is still lacking to use PPFM a biocontrol agent. The first report in our laboratory on induction of systemic resistance in plants sprayed with PPFM having ultimate effect on reduced leaf incidence (Madhaiyan *et al*., 2004 and 2006) have provoked us to use PPFM as a biocontrol agent to control root rot of cotton caused by *Rhizoctonia solani*.In the present investigation, we have tested the antagonistic potential of many methylobacterial strains already isolated in our laboratory against *R.solani* in dual culturing method on Potato dextrose solid and liquid media. The result clearly indicated that *Methylobacterium extorquens* CO 47 showing maximum suppression of the mycelial growth of *Rhizoctonia solani* under *in vitro* conditions.

Studies on induction of defense related proteins in cotton plants due to inoculation of *Methylobacterium extorquens* CO47 and *Rhizoctonia solani*

Peroxidase (PO):

The increase in peroxidase activity of leaves up to 5th day of inoculation and later a slow decline was noticed. PPFM inoculation registered better activity when compared to control. Maximum activity was observed with the inoculation of PPFM and *R solani* (3.51 abs min^{-1} g^{-1} tissue) which was significantly higher than other treatments (Fig. 1). Peroxidase represents A aomponent of an early response in plants to pathogen attack and plays key role in the biosynthesis of lignin, which limits the extent of pathogen spread (Bruce and West, 1989). Increased peroxidase (PO) has been observed in a number of resistant interaction involving plant pathogenic fungi, bacteria and virus (Nandakumar *et al*., 2001)

Polyphenol oxidase (PPO):

The cotton plants expressed higher activity of PPO

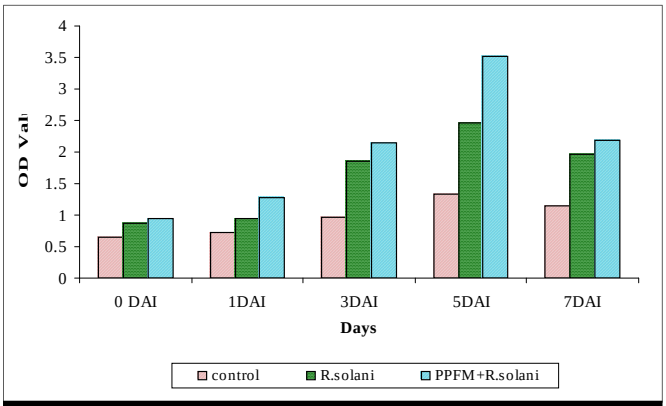

Fig. 1 : Induction of PO activity in cotton treated with *Methylobacterium extorquens* CO 47

when treated with PPFM CO 47 challenged with *Rhizoctonia solani* till 5th day after challenged inoculation (2.32 abs min^{-1} g^{-1} tissue) and there after a slow decline (1.94 abs min^{-1} g^{-1} tissue) (Fig. 2). Madhaiyan *et al*. (2004) have well documented the capability of *Methylobacterium* to induce the defence related activities of polyphenol oxidase, peroxidase, chitinase**,** β-1, 3-glucanase and phenylalanine ammonia lyase in response to *R.solani* infection in rice plants.

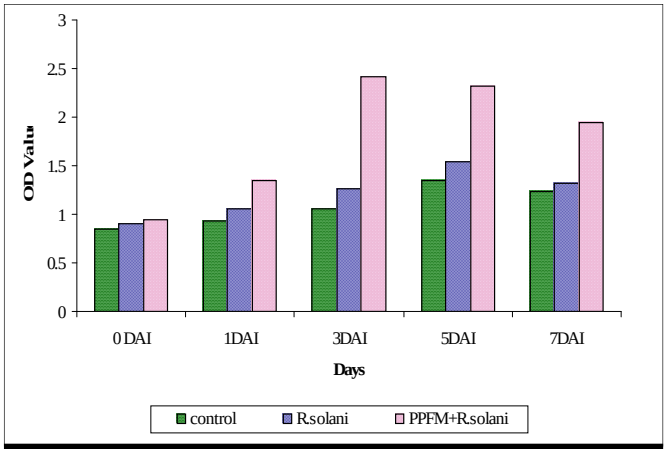

Fig. 2 : Induction of PPO activity in cotton treated with *Methylobacterium extorquens* CO 47

Phenylalanine lyase (PAL):

Phenylalanine ammonia lyase plays an important role in the biosynthesis of phenolic phytoalexins. The increase in PAL activity indicates the activation of phenyl propanoid pathway. In the estabilishment phase of a pathogen with in the host tisues, PAL activity often increases (Fig. 3). In several host pathogen interactions, increased levels have been shown to be correlated with incompatibility (Battacharya and Ward, 1988). The phenylalanine lyase activity was found to be increased from 1st day and remain

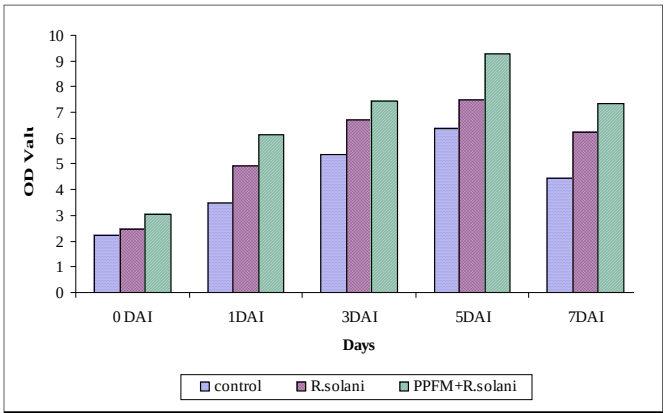

Fig. 3 : Induction of PAL activity in cotton treated with *Methylobacterium extorquens* CO 47

high till 5th day after inoculation. Among the treatments soil application with PPFM CO 47 and challenge inoculation with *Rhizoctonia solani* recorded the maximum PAL activity 9.27 µ mol of cinnamic acid /min/ g of leaf tissue on 5th day after challenge inoculation which is found to be high compared to inoculated control 6.39µ mol of cinnamic acid /min/g of leaf tissue.

Phenol:

Phenols play an important role in determining resistance or susceptibility of a host to parasite infection (Vidhyasekaran, 1998). Lignin is the phenolic polymer which is difficult to be breached by pathogen and has been implicated in plant defense against pests and diseases (Nicholson and Hammerschmidt, 1992). In cotton plants inoculated with, PPFM CO 47 and challenged with pathogen, the total phenol content was 518µg g^{-1} fresh tissue on 5th day after challenge inoculation.The result clearly indicated that accumulation of phenolic compounds was comparatively much lower (274 µg g^{-1} fresh tissue) in pathogen inoculated control when compared to PPFM CO47 treated plants and challenged with pathogen (Fig. 4). Reddy (2002) earlier reported that due to inoculation of *Methylobacterium*, peroxidase, β-1, 3-glucanase, PAL and poly phenoloxidase increased in groundnut plant challenge inoculated with *Sclerotium rolfsii* and *Aspergillus niger.* The PPFM strain with induced systemic resistance activity was previously attributed for achieving a better disease suppression. The considerable evidences reported already in understanding the plant defence mechanism have given clues on the possible contributing biocontrol effect of PPFM in cotton plants.

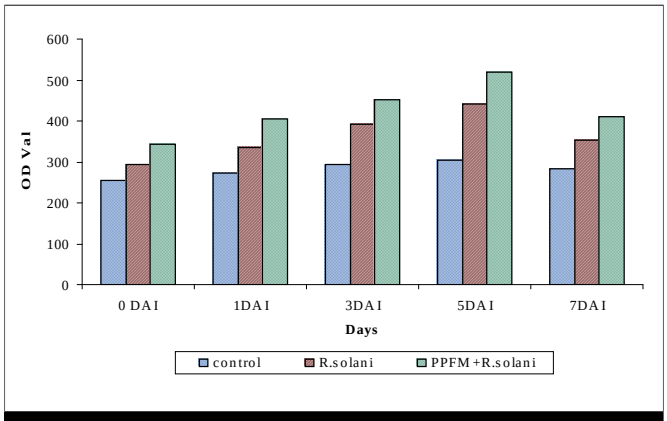

Fig. 4 : Induction of phenol in cotton treated with *Methylobacterium extorquens* CO 47

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REFERENCES

Battacharya, M.K. and Ward, E.W.B. (1988). Phenylalanine ammonia lyase activity in soybean hypocotyls and leaves following infection with *Phytophthora megasperma* f. sp. *glycinea*. *Canadian J. Bot.*, **66** : 18-23.

Blakeman, J.P. and Fokkema, N.J. (1982). Potential for biological control of plant diseases on the phylloplane. *Annu. Rev. Phytopathol.*, **20** : 167-192.

Bruce, R.J. and West C.A. (1989). Elicitation of lignin biosynthesis and isoperoxidase activity by pectic fragments in suspension cultures caster bean. *Pl. Physiol.*, **91**: 889-897.

Cheng, G.Y., Zheng T.M. and Mow Q.Y. (1993). Experiments on the control of apple leaf and fruit diseases with fluorescent *Pseudomonas* sp. *Chinese J.Biol.Control.*, **9** : 163-166.

Dennis, C. and Webster , J. (1971). Antagonistic properties of species groups of *Trichoderma*. I. Production of non-volatile antibiotics. *Trans. Br. Mycol. Soc.*, **57** : 25-39.

Dickerson, D.P., Pascholati, S.F, Hagerman, A.E., Butler, L.G and Niholson, R.L. (1984). Phenylalanine ammonia lyase and hydroxyl cinnamate: CoA ligase in maize mesocotyls inoculated with *Helminthosporium maydis* or *Helminthosporium carbonum. Physiol. Pl. Pathol.*, **25** : 111-123.

Green, P.N. (1992). The genus *Methylobacterium*. In: *The prokaryotes*, 2nd Ed. (eds.) A. Baloes, H.G. Truper, M. Dworkin, W. Harder, and K.H. Schleifer. Springer-Verlag, Berlin, pp. 2342-2349.

Green, P.N and Bousifield I.J. (1981). The taxonomy of pink-pigmented facultatively methylotrophic bacteria, In: *Microbial Growth C1 Compounds*. (eds.)Dalton H Heyden and Son Ltd . London. pp. 285-293.

Green, P.N. and Bousifield, I.J. (1983). Emendation of *Methylobacterium* Patt, Cole and Hanson 1976, *Methylobacterium rhodinum* (Heumann 1962) comb. Nov.corig; *Methylobacterium radiotolerans* (Ito and Iizuka 1971), comb.nov.corrig., and *Methylobacterium mesophilicum* (Austin and Goodfellow 1979) comb.nov. *Internat. J. Syst. Bacteriol.,* **33** : 875.

Hammerschmidt, R., Nuckles, E.M. and Kuc, J. (1982). Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. *Physiol. Pl. Pathol.,* **20** : 73-82.

Madhaiyan, M., Poonguzhali, S., Senthilkumar, M., Seshadri, S., Chung, H., Yang, J. Sundaram, S.P. and Tongmin, S.A. (2004). Growth promotion and induction of s.ystemic resistance in rice cultivar Co-47 (*Oryza sativa* L.) by *Methylobacterium* spp. *Bot Bull. Acad. Sin.,* **45:** 315-324.

Madhaiyan, M., Suresh Reddy, B.V., Anadham, R., Senthilkumar, M. Poonguzhali, S., Sundaram, S.P. and Tongmin, S.A. (2006). Plant growth promoting *Methylobacterium* induces defence response in groundnut compared with rot pathogens. *Curr. Microbiol.,* **53** : 270–276.

Mayer, A.M., Harel, E. and Shaul, R.B. (1965). Assay of catechol oxidase a critical comparison of methods. *Phytochem.,* **5** : 783-789.

Nandakumar, R., Babu, S., Viswanathan, R., Raguchander, T. and Samiyappan, R. (2001). Induction of systemic resistance in rice against sheath blight disease by plant growth promoting rhizobacteria. *Soil Biol. Biochem.,* **33** : 603-612.

Nicholson, R.L. and Hammerschmidt, R. (1992). Phenolic compounds and their role in disease resistance. *Annu. Rev. Phytopathol.,* **30** : 369-389.

Reddy, Suresh (2002). Studies on pink pigmented facultative methylotrophs as a new bioinoculant for groundnut (*Arachis hypogaea* L.), M.Sc. (Ag.)Thesis, Tamil Nadu Agricultural University, Coimbatore.

Vidhyasekaran, P. (1998). Physiology of Disease resistance in plants. Vol. I. CRC. press. Boca Raton, FL. p.149.

Zieslin, N and Ben-Zaken, R. (1993). Peroxidase activity and presence of phenolic substances in peduncles of rose flower. *Pl. Physiol. Biochem.,* **31**: 333 – 339**.**

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