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Field Evaluation of Chitosan and *Pseudomonas* Sp. on the Biological Control of Tomato Leaf Curl Virus (Tolcv) in Tomato

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Application of *Pseudomonas* spp.in combination with chitosan reduced the severity of ToLCV diseased plants by 48% at 45 DAI. Application of chitosan or the bacterial inoculant alone was not very effective. The plants inoculated with the combination of *Pseudomonas* sp. 206(4) and Chitosan recorded the highest phenol content, peroxidase, polyphenol oxidase, chitinase and PALase activities. This treatment also recorded maximum plant height, biomass and chlorophyll content, number of fruits per plant, fruit yield and shelf life of fruits over the diseased control. The semi quantitative PCR analysis revealed the lowest viral load accumulation in plants inoculated with 206(4) and Chitosan. Thus, the study has indicated that the application of *Pseudomonas* sp. 206(4) and Chitosan effectively reduced the disease severity of ToLCV through ISR as evidenced by lower viral titre and higher production of defense molecules.

Key words: *Pseudomonas* spp., ToLCV, tomato, Chitosan, biocontrol, *Bemisia tabaci*.

Tomato leaf curl virus (Tolcv), a geminivirus, is the most important and destructive viral pathogen in many parts of India (Saikia and Muniyappa, 1989). The incidence of ToLCV in tomato growing areas of Karnataka ranged from 17 to 100 per cent in different seasons (Saikia and Muniyappa, 1989). The disease is transmitted by whitefly *Bemisia tabaci* (Gennadius) (Homoptera : Aleyrodidae) (Muniyappa and Veeresh, 1984).

Several approaches have been attempted to manage the tomato leaf curl virus. Vector control through spray of chemicals in many instances does not bring about the desired results. Commercially acceptable resistant varieties are not always available. Of late, management of virus diseases has been attempted by many scientists using plant growth promoting rhizobacteria (PGPR) as an alternative strategy for the disease management which is ecologically sound and environmentally safe.

The investigation of plant response to elicitiors is one of the most rapidly developing lines of inquiry in plant physiology. The elicitiors stimulate the contact between plant-phytopathogens and thereby trigger defensive mechanisms that constrain the invasion of pathogenic fungi, bacteria and viruses. Chitosan is one of the most studied elicitiors. It regulates the expression of resistance genes and induces jasmonate synthesis (Doares *et al*,1995).Fragments from chitin and chitosan are known to have eliciting activities leading to a variety of defense responses in host plants in response to microbial infection, inducing the accumulation of phytoalexin, pathogenesis related (PR) protein and proteinase inhibitors, lignin synthesis and callose formation. Chitin and chitosan are naturally-occurring compounds that have potential in agriculture in controlling plant diseases. They have been reported

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to be active against viruses, bacteria and other pests (Abdelbasset *et al.* ,2010). Based on these and other proprieties that help strengthen host plant defenses, interest has been growing in using them in agricultural systems to reduce the negative impact of diseases on yield and quality of crops.

Several antagonistic microorganisms such as *Pseudomonas, Bacillus, Streptomyces, Gliocladium and Trichoderma* spp. have the potential to control a variety of crops diseases. However, effects of single biocontrol agents were often insufficient under practical conditions or difficult to reproduce (Punja and Yip, 2003). In other studies, beneficial microorganisms were combined with a natural compound such as chitin (Sid-Ahmed et al., 2003) or chitosan (Benhamou et al., 1998) to improve their biocontrol efficacy. Hence, the present study was conducted to assess the biocontrol efficacy of PGPR formulation supplemented with chitosan to control ToLCV in field conditions and elucidate the mechanism of biocontrol.

MATERIALS AND METHODS

Field study

A field experiment was conducted to assess the effect of selected PGPR strains and chitosan on the disease severity control as well as on growth and yield of tomato. It was carried out at Main Agricultural Research Station, U A S, Dharwad, Summer (Jan- June '2012). Five weeks old seedlings of variety Pusa Ruby, raised in a glasshouse were transplanted in the main field with plot size 13m x 10 m and 75cm x 60cm spacing. In the chemical control treatment, confidor @2 ml/L was sprayed at weekly intervals to control the vector, as per the package of practices for tomato crop.

Rhizobacterial and chitosan treatment

Bacteria were grown in King's B broth medium on a shaker(150 rpm) for 2 days and centrifuged at 10,000 rpm for 5 min. Chitosan was dissolved in 100 mM acetate buffer (pH 4.5) and the pH adjusted to 6.5 using 1 N NaOH. The cell pellet was mixed with chitosan solution(5%). Tomato seeds of the variety, Pusa Ruby (susceptible to ToLCV) were used in the experiment. They were surface sterilized with sodium hypochlorite solution were soaked in chitosan cell suspension and kept at shaking condition for 3 hrs at 28°C. The seeds were shaken in chitosan solution until they became fully coated. The biocoated seeds were dried inside a laminar flow chamber. For soil application, the lignite based culture (1:3) was applied to soil @ 5kg/ha before sowing seeds and mixed well. For foliar application, the lignite based culture was filtered through a muslin cloth and sprayed @ 1% (w/v) at 10 days after sowing (DAS) and 20 DAS. At 25 DAS ,both upper and lower surfaces of the leaves were sprayed with the Chitosan solution (1 mg/ml) prepared in 100 mM acetate buffer (pH 4.5) and adjusted with 1 N NaOH to pH 6.5.Control plants in pots without application of rhizobacteria and chitosan were also maintained. All treatments were replicated eight times and arranged in a randomized complete block design (RCBD).

ToLCV inoculation

Whiteflies were collected from cotton and tobacco plants from fields by sucking with the help of an aspirator by slowly turning the leaves slightly upwards. Whiteflies were released on to the ToLCV diseased tomato plants grown in insect proof rearing cages and continuously maintained by introducing younger plants in to the rearing cage ,thus, making insects viruliferous. The viruliferous insects were sucked from the diseased plants with the help of an aspirator and released on to healthy, rhizobacteria treated tomato seedlings on the top leaves. Immediately, the seedlings were placed in an insect proof rearing cage and allowed insects for a week to feed on them and bring about infection by the virus. Thirty days old seedlings were used for release of the viruliferous insects . Thus, it was ensured that all seedlings were infected with ToLCV.

Sample collection, Enzyme and phenol estimation

Leaf samples were collected at 45 DAS and 75 DAS from both inoculated and uninoculated tomato plants. They were frozen immediately in liquid nitrogen, ground to a powder and stored at -80° C until determination of phenylalanine ammonia lyase (PALase), chitinase, polyphenol oxidase and peroxidase activities.

The Peroxidase activity was assayed spectrophotometrically following the method described by Mahadevan and Sridhar (1986). The PALase activity was determined using the method described by Ross and Sederoff (1992). The

polyphenol oxidase activity in leaves was estimated at 45 and 75 DAS following the method of Mayer *et al*. (1965). The chitinase activity was estimated at 45 and 75 DAS, following the method described by Ross and Miller (1959).The total phenol content in leaves was estimated at 45 and 75 DAS by following Folin Cio-calteau method (Sadasivam and Manickam, 1991).

Confirmation of ToLCV particles in affected leaves

The transformed cells which has taken ToLCV coat specific gene insert were picked up and streaked on Luria agar containing ampicillin (100 µg/ml). The plasmids were isolated from clones and the clones were confirmed through PCR amplification (Fig 1a.) by using specific primers. Further, confirmation was also done by restriction analysis (Appendix III) using *Eco*RI / *Hind*III, which released app.1035 bp (Fig 1b.)

The clone was selected for sequencing. The insert in PTZ57R/T was sequenced using M13 F/R primer at Ocimum Biosolutions Ltd., Hyderabad. Homology search was done with BLAST algorithm available at http://www.ncbi.nlm.nih.gov. The insert fragment were sequenced and subjected to BLAST search at NCBI, which showed 98% similarity with Tomato Leaf Curl Banglore Virus Isolate (ToLBV-AVT1 segment
DNA A)

Monitoring of the disease

Disease severity (% diseased plants severly affected) and symptoms severity were recorded according to the disease severity scale described by Muniyappa *et al.,* (1991).

The viral disease was monitored in all the treatments. Observations were made at 30, 45 and 75 days after viral inoculation (DAI) for ToLCV symptoms on tomato and the percent disease severity calculated using the formula,

$$\text{Percent disease severity} = \frac{\text{No. of plants severely infected (SI)}}{\text{Total number of plants}} \times 100$$

Semi quantitative analysis of rhizobacterial treated leaves

Semi quantitative PCR was carried out for detection and estimation of the viral DNA accumulation in the leaves. One hundred nanogram of DNA from each treatment was used as template in a 20µl PCR reaction containing PCR ingredients. For the PCR reaction, dNTP (1 mM), Forward (5 pM) and Reverse (5pM) primer, Taq buffer and Taq polymerase were used. The PCR reaction was performed for different reaction cycles('Eppendorf' make Thermal cycles) of 10, 20 and 30 cycles with the same reaction conditions throughout. After the reaction, the samples were run on 1% agarose gel for comparison. ToLCV specific coat protein primers - forward sequence - 5' GGT CCC CTC CAC TAA ATCAT 3'(20nt) and reverse sequence 5'-5'CAG TTG GTT ACA GAA TCG TAG AAG 3'(24nt) were used for the amplification of the coat protein of ToLCV.

Growth and Physiological parameters

The influence of rhizobacteria on growth of tomato plants was assessed. The plant height, total biomass content, fruit number and fruit weight per plant, shelf life of tomato fruits and chlorophyll content were made periodically. Chlorophyll content was measured by using a SPAD (Soil Plant Analysis Device) meter by selecting four leaves randomly at the centre of the branch and the average worked out.

Statistical analysis

The data obtained from field experiments were subjected to Randomized Complete Block Design analysis as described by Gomez and Gomez (1984). The level of significance used in the 'F' test was P=0.05. The critical difference values were calculated whenever the F test values were significant.

RESULTS AND DISCUSSION

Early and accurate diagnosis of plant viruses is a key component of any crop disease management system. Since the biological methods of viral detection are too slow and not amenable to large scale application, molecular biology tools are being applied for rapid, specific and sensitive detection of viral pathogens (Miller and Martin, 1988). The increasingly popular use of PCR and RT-PCR for the diagnosis of plant viruses is appreciated due to highly sensitive and reliable methods for viral nucleic acid detection (Thomson and Dietzgen, 1995).

In order to rule out the possibility of amplification of off-targets, the PCR product of ToLCV were cloned in PTZ57R/T cloning vector and sequenced. The resulted sequence showed 98 per cent homology with Tomato leaf curl Bangalore virus isolate ToLCBV-AVT1 segment

DNA A , confirming the ToLCV disease of tomato.

The biocontrol ability of rhizobacterial isolates alone, in mixture, or in combination with chitosan was evaluated under high virus - vector pressure conditions previously. Based on the previous experiment, one best bacterial isolate 206 (4) along with chitosan was selected for further characterization and biocontrol studies. These results provide evidence that PGPR-mediated induced resistance against ToLCV on tomato, previously reported from greenhouse experiments (Shefali *et al.,* 2012) and confirmed here, can be obtained under field conditions. It is known that virus symptoms and concomitant negative effects on yields are most severe when plants are infected with virus in early growth stages (Matthews *et al.,* 1991). In the, field experiment, the incidence of ToLCV infection was lower on PGPR and chitosan treated plants. Application of this *Pseudomonas* sp. 206(4) along with chitosan significantly reduced ToLCV severity, achieving a disease severity reduction of 48 per cent at 45 DAI and 24 per cent at 75 DAI (Fig 2a and 2b). Vasanthi *et al* (2010) also observed that tomato leaf curl virus infected plants were significantly lower (25%) with less symptom severity and delayed symptom expression in *Pseudomonas* sp. VPT10 with chitin treated tomato plants as compared to non-bacterized control plants. Higher biocontrol efficacy of *P. fluorescens* against CMV and tomato mottle virus in tomato under field conditions was reported by Murphy *et al.* (2000).

Recent investigations on the mechanisms of biocontrol by rhizobacteria have revealed that several strains protected plants from various pathogens including viruses by activating defense molecules (Kandan *et al*., 2002). In the present study ,rhizobacteria controlled ToLCV essentially through induced systemisc resistance. Plants inoculted with 206(4) and Chitosan have registered the highest level of phenolics (11.53 per cent higher than in the disease control) (Table 1) and defense enzymes compared to other treatments. Similarly, Kandan *et al*. (2003), also

Table 1. Effect of the best selected rhizobacteria and chitosan on defense molecules activity

Treatments	Phenol (mg/g dry weight)	Peroxidase (ΔOD/g protein/ min)	Chitinase (µgGlc NAc/ µg protein/ min)	Polyphenol oxidase (ΔOD/g protein /min)	Phenyla-mmonia lyase (changes in cinnamic acid/min/g)
Pseudomonas 206(4) + Chitosan +ToLCV	0.26	1.51	2.65	1.37	0.184
Chemical control +ToLCV	0.21	1.14	1.74	0.93	0.148
Diseased control (only ToLCV)	0.23	1.26	1.96	1.02	0.164
SEm+	0.01	0.07	0.09	0.04	0.02
CD @5%	0.02	0.21	0.26	0.12	0.05

Table 2. Plant growth parameters of tomato as influenced by inoculation with rhizobacteria and chitosan (At harvest)

Treatments	Plant height (cm)	Total biomass (g/plant)	Chlorophyll (SPAD)	Fruit yield (kg/plant)	Number of fruits per plant	Shelf Life (days)
Pseudomonas 206(4) + Chitosan +ToLCV	31.1	31.04	20.7	0.207	12.2	5.4
Chemical control +ToLCV	26.9	23.94	17.5	0.092	6.4	4.7
Diseased control (only ToLCV)	20.3	13.02	10.6	0.058	2.6	4.4
SEm+	0.59	0.57	1.21	0.01	0.47	0.24
CD @5%	1.81	1.75	3.73	0.03	1.45	0.75

observed increased phenolics in cowpea due to *P. fluorescens* inoculation which, in turn, protected plants from spotted wilt virus. Inoculation of tomato plants with 206(4) and chitosan also resulted in the highest synthesis of phenylalanine ammonia lyase (10.86 per cent higher than the diseased control) (Table 1). Phenylalanine ammonia lyase (PALase) activity has been observed to be induced during plant -pathogen and plant - pest interactions (Harish, 2005) and is known to play an important role in the biosynthesis of various defense chemicals in phenyl propanoid metabolism (Daayf *et al.,* 1997). Though all the treatments induced biosynthesis of peroxidase, the 206(4) + Chitosan showed the highest peroxidase activity which was 16.55 per cent more than the diseased control (Table 1). Liana et al (2011) succeeded in controlling tobacco mosaic virus by *Bacillus* sp. through induced systemic resistance in tobacco as evidenced by increased levels of defense enzymes such as phenylalanine ammonia-lyase, peroxidase,polyphenol oxidase and pathogenesis-related (PR) proteins in tobacco .

Another fascinating observation was the enhanced chitinase activity in rhizobacteria and chitosan treated plants (26.03 per cent higher than

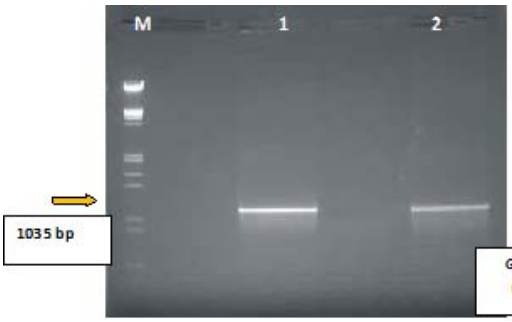

Fig. 1(a). PCR confirmation of ToLCV coat specific gene

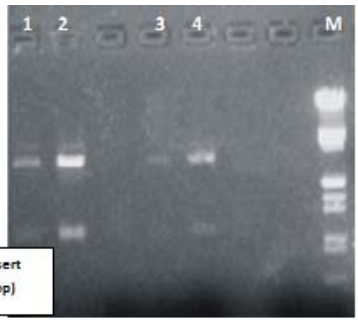

Fig. 1(b). RFLP confirmation of ToLCV coat specific gene

Fig. 2(a). *Pseudomonas* sp. 206(4)+ Chitosan Chemical control Disease control

the diseased control) (Table 1). This increase in chitinase activity might have prevented the damage caused by viral pathogen and, thus, reduced the disease severity. Synthesis and accumulation of PR proteins have been implicated in plant defence mechanisms. Chitinases, which are classified under PR-3 have been reported to associate with resistance in plants against pests and diseases (Maurhofer *et al.*, 1994 and Van Loon, 1997). Inoculation of tomato plants with 206(4) and chitosan also resulted in the highest synthesis of polyphenol oxidase activity (25.54 per cent higher

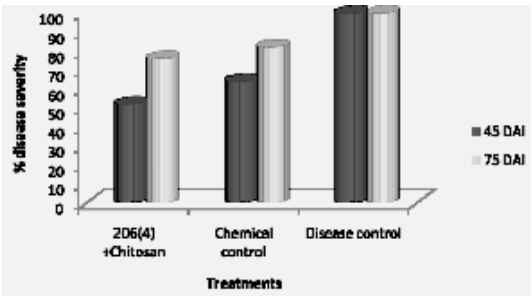

Fig. 2(b). Biocontrol of ToLCV disease by the *Pseudomonas* sp. 206(4) +Chitosan

than the diseased control) (Table 1). PPO has been shown to be inhibitory to viruses by inactivating the RNA of the virus (Vidhyasekaran, 1988 a). Nandeeshkumar *et al* (2008) observed enhanced activation of catalase, PAL, Peroxidase, PPO and chitinase level in sunflower when seeds were treated with 5 per cent chitosan for controlling downy mildew. Yu *et al* (2007) found that combination of chitosan and *Cryptococcus laurentii* resulted in a synergistic inhibition of the blue mold rot caused by *Penicillum expansum* in apple. Similar kind of inhibition was reported on tomato leaves treated with chitosan and challenge inoculated with potato spindle tuber viroid (Pospiezny,1997).

The semi quantitative PCR can be used to detect and estimate the viral DNA load in plants. Hence, PCR reactions using coat protein gene specific primers were performed for different number of cycles *viz.*, 10, 20 and 30. The leaves of rhizobacteria-treated and untreated tomato plants were tested for the presence of the virus. At both 45 DAS and 75 DAS, 206(4) and chitosan treated plants showed the least viral load (Fig 3). However, this needs to be further validated. Zehnder *et al* (2000) followed ELISA method to detect the viral load (cucumber mosaic virus) in PGPR treated cucumber plants. Even in field trials, they observed

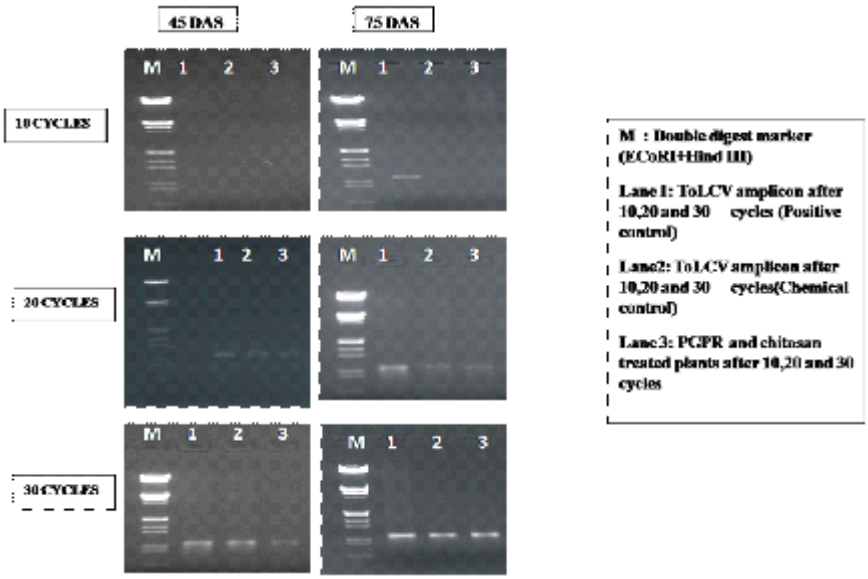

Fig. 3. Semi-quantitative PCR analysis of ToLCV in tomato
(The amplicon size of ToLCV coat specific gene insert is 1035 bp)

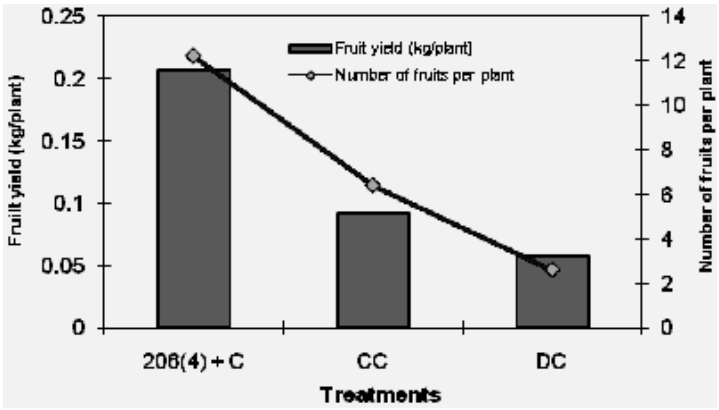

Fig. 4. Influence of the best selected rhizobacterial isolate and chitosan on yield of tomato

significantly lower ELISA values in all PGPR treatments than in the disease control, with a concomitant decrease in disease severity. Liana *et al* (2011) also observed a decreased amount of tobacco mosaic virus load in *Bacillus* strain EN16- or SW1-treated tobacco plants through ELISA.

In addition to suppressing the viral disease, 206(4) and chitosan greatly improved plant growth, biomass, chlorophyll content and yield (Table 2, Fig.4). This may be due to higher production of phytohormones by the rhizobacteria. *Pseudomonas* sp. B-40 had stimulated growth and yield of tomato in earlier investigations (Earnapalli 2005; Kirankumar, 2007). Inoculation of 206(4) and chitosan increased the plant height by 34.72 percent, biomass by 58.05 percent and chlorophyll content by over 48.79 compared to disease control. The percent increase in fruit yied by 71.98 percent, number of fruits per plant by 78.68 per cent and 18.51 percent increase in shelf life of tomato fruits were observed compared to the virus inoculated control. Chitin supplemented peat formulation of *Bacillus subtilis* AF1 increased the emergence and dry weight of pigeon pea seedlings by 29 and 33 per cent, in comparision to an increase of 21 and 30 per cent, respectively by *Bacillus subtilis* AF1alone (Manjula and Podile , 2005).

Thus, the study has clearly brought out that *Pseudomonas* sp. 206(4) and chitosan was more effective in reducing the disease severity of ToLCV under field condition through ISR besides plant growth promotion.

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REFERENCES

1. Abdelbasset, E., Hadrami, Adam, L. R., Hadrami, I. E. , Daayf, F. Chitosan in Plant Protection. *Mar. Drugs*.,2010; **8**: 968-87.
2. Benhamou, N. , Kloepper, J . W. S. Induction of systemic resistance to Fusarium crown and root rot in tomato plants by seed treatment with chitosan. *Planta*.,1998; **204** :153–68.
3. Doares, S. H., T., Weiler, E. W., Ryan, C. A. Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. Proc National academy Science, USA.,1995; **92**: 4095-98
4. Daayf, F., Bel – Rhlid, R., Belange, R. R . Methyl ester of P- coumaric acid: A phytoalexin like compound from long English cucumber leaves. *J. Chem. Ecol*.,1997; **23**: 1517-26.
5. Earnapalli, V. N. Screening of antagonistic microorganisms for biological control of early blight of tomato caused by *Alternaria solani*, M.Sc. thesis., University of Agricultural Sciences, Dept. of Agricultural Microbiology, Dharwad (India), 2005.
6. Harish, S. Molecular biology and diagnosis of banana bunchy top virus and its management through induced systemic resistance Ph.D. thesis., Tamil Nadu Agricultural University, Coimbatore, India, 2005.
7. Kandan, A., Radjacommare, R., Nandakumar, R. , Raghuchander, T., Ramiah, M., Samiyappan, R. *Folia Microbiol.*,2002; **47**(2): 121-29.
8. Kandan, A., Radjacommare, R., Ramiah, M., Ramanathan, A., Samiyappan, R. In: Proceedings of the Sixth International Workshop on Plant Growth Promoting Rhizobacteria, Ed. Y.R. Sarma, IISR Publishers, Calicut,2003; pp 480-86.
9. Kavino, M., Harish, S., Kumar, N., Soorianathasundaram, K., Ramanathan, A., Samiyappan, R. In: Proceedings of the Sixth International Workshop on Plant Growth Promoting Rhizobacteria, Ed. Y.R. Sarma, IISR Publishers, Calicut,2003; pp. 486-92.
10. Kirankumar, R. Evaluation of Plant growth promoting rhizobacterial stains against TMV on tomato. M. Sc Thesis,2007; University of Agricultural Sciences, Dept. of Agricultural Microbiology. Dharwad (India).
11. Kim,Y., C. , Jung, H. , Kim, K. Y., Park, S. K. An effective biocontrol bioformulation against Phytophthora blight of pepper using growth mixtures of combined chitinolytic bacteria under different field conditions. *Eur. J. Plant Pathol*., 2008; **120**: 373–82.
12. Liana, L. C., Liyan, X. B., Luping, Z. B., Qiying, L. B. Induction of systemic resistance in tobacco against Tobacco mosaic virus by *Bacillus* spp. *Biocont. Sci. Technol*., 2011; **21**: 281-92.
13. M'Piga, Belanger, R. R., Paulitz, T .C., Benhamli, N. Increased resistance to tomato plants treated with endophytic bacterium *Pseudomonas fluorescens* .*Physiology and Molecular Plant Pathology*.,1997; **50** : 301-20.
14. Maurhofer, M. , Hase, C. , Meuwly, P. , Metraux, J. P. , Defago, G. Induction of systemic

resistance of tobacco to tobacco necrosis virus by the root colonizing *Pseudomonas fluorescens* strain CHAO : Influence of the gacA gene and of pyoverdine production. *Phytopathol.*,1994; **84**: 139-46.

15. Muniyappa , V., Veeresh, G . K. Plant virus diseases transmitted by whiteflies in Karnataka. *Proceedings of Indian Academy of Sciences*,1984; **93**: 397-406.
16. Muniyappa, V., Jalikop, S. H., Saikia, A. K., Channarayappa,K., Shivashankar, G., Ishwarabhat, A. , Ramappa, H .K. Reaction of *Lycopersicon* cultivars and wild accessions to tomato leaf curl virus. *Euphytica*.,1991;**56**: 37-41.
17. Mahadevan A., Sridhar R. Methods in Physiological Plant Pathology. Sivakami Publishers, Madras, 1986; pp 103-08.
18. Murphy, J .F., Zehnder, G. W., Suhuster, D. J., Sikora, J. E., Polston., Kloepper, J. W. Plant growth promoting rhizobacterial mediated protection in tomato against tomato mottle virus. *Plant Dis.*,2000; **84** :779-84.
19. Manjula, K., Podile, A. R. Increase in seedling emergence and dry weight of pigeon pea in the field with chitin supplemented formulations of *Bacillus subtilis* AF1. *W. J. Microbiol. Biotechnol*., 2005; **21**: 1057-62.
20. Miller, G. L.Use of dinitrosalicyclic acid reagent for determination of reducing sugar. *Analytical Chem.*,1959; **31** : 426-28.
21. Miller, S. A., Martin, R. R. Molecular diagnosis of plant diseases. *Ann. Rev. Phytopathol*., 1988; 26:409-32.
22. Matthews, R.E.F. *Plant virology,* 3rd Edition. Academic Press, San Diego, California., 1991; pp 177.
23. Mayer, A. M., Harel, E., Shaul, R. B. Assay of Catechol oxidase: a critical comparison of methods. *Phytochem.*, 1965; **5** :783.
24. Nandeeshkumar, P., Sudisha, J., Ramachandra, K. K., Prakash, H. S., Niranjana, S. R., Shekar, S. H. Chitosan induced resistance to downy mildew in sunflower caused by *Plasmopara halstedii*. *Physio. Mol. Plant Pathol*.,2008; **72**: 188-94.
25. Pospieszny, H. Antiviroid activity of chitosan. *Crop Prot.*,1997; **16** : 105–06.
26. Punja, Z. K., Yip, R. Biological control of damping-off and root rot caused by *Pythium aphanidermatum* on greenhouse cucumbers. *Can. J. Plant Pathol.*,2003; **25**: 411–17.
27. Raupach, G. S., Kloepper, J. W. *Phytopathol.*, Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. 1998; **88**: 1158–64.
28. Ross, W. W., Sederoff, R. R. Phenylalanine ammonia lyase activity from lobally pine: Purification of the enzyme and isolation of complementary DNA clones. *Plant Physiol.*,1992; 98 : 380-86
29. Sadasivam ,S., Manickam, A. Biochemical Methods for Agriculture Science, Wiley Eastern Limited, New Delhi,1991; pp 106-108.
30. Saikia, A. K., Muniyappa, V. Epidemiology and control of tomato leaf curl virus in *Southern India*. *Trop Agric*.,1989; **66**: 350–54.
31. Srinivasan, K., Surendiran, G., Maathivanan, N. Asian Conference on Emerging Trends in Plant-Microbe Interaction, 8-10 December,2005; Chennai, India.
32. Sid-Ahmed, A., Ezziyyani, M., Pérez- Sánchez, C., Candela, M. E. Effect of chitin on biological control activity of *Bacillus* spp and *Trichoderma harzianum* against root rot disease in pepper (*Capsicum annuum*) plants. *Eur. J.Plant Pathol*.,2003; **109** :633–37.
33. Shefali, M. Plant growth promoting rhizobacteria-induced systemic resistance against ToLCV disease in tomato (*lycopersicon esculentum* mill.). Ph.D Thesis,2012; University of Agricultural Sciences, Dept. of Agricultural Microbiology. Dharwad (India).
34. Thomson, D., Dietzgen, R. G. Detection of DNA and RNA plant viruses by PCR and RT-PCR using rapid virus release protocol without tissue homogenization. *J. Virol Method*.,1995; **54**: 85-95.
35. Vasanthi, V. J., Kandan, A., Ramanathana, A., Raguchandera, T., Balasubramaniana, P., Samiyappana, R. Induced systemic resistance to tomato leaf curl virus and increased yield in tomato by plant growth promoting rhizobacteria under field conditions. *Arch. Phytopathol. Plant Prot.*,2010; **43**(15): 1463–72.
36. Van Loon, L. C. Induced resistance in plants and the role of pathogenesis related proteins. *European J.* of *Plant Pathol.*, 1997; **103:** 753-65.
37. Vidhyasekaran, P. Physiology of disease resistance in plants. Vol ™. Boca Raton, F L: CRC Press.,1988.
38. Yu, T., Hong, Y. L., Xiao, D. Z. Synergistic effect of chitosan and *Cryptococcus laurentii* on inhibition of *Penicillium expansum* infections. *Int. J. Food Microbiol.*, 2007; **114**: 261–66.
39. Zehnder, G .W., Yao, C., Murphy, J .F., Sikora, E. R., Kloepper, W. Induction of resistance in tomato against cucumber mosaic cucumovirus by plant growth promoting rhizobacteria. *Biocontrol*.,2000; **45**: 127-37.