JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Dec. 2014. Vol. **8**(6), p. 4749-4757

Subcellular Changes in Tomato Leaves during Induced Resistance Elicited by Rhizobacteria and Chitosan against Tomato Leaf Curl Virus (ToLCV) under Field Conditions

Shefali Mishra[1]*, K.S. Jagadeesh[1] and P.U. Krishnaraj[2]

[1]Department of Agricultural Microbiology, [2]Department of Biotechnology. College of Agriculture, Dharwad, University of Agricultural Sciences, Dharwad - 580 005, India.

(Received: 15 July 2014; accepted: 10 September 2014)

High field incidence of diseases caused by insect transmitted viruses pose serious epidemic of ToLCV on tomoato, where the insecticides completely ineffective to preventing in the spreading the virus. Application of rhizobacterial mixture in combination with chitosan reduced the severity of ToLCV (Tomato leaf curl virus) diseased plants by 83.6–93.3 per cent. The plants inoculated with the chitosan based formulation of *Pseudomonas* sp.(206(4) +B-15+ JK-16) showed concomitant increase in plant height, total biomass, chlorophyll content, fruit yield and shelf life over the diseased control. The quantification of the virus, as done through semi quantitative PCR analysis, revealed the least viral load accumulation in plants inoculated with both chitosan and rhizobacterial mixture. The subcellular changes inside the cells, as studied by transmission electron microscopy revealed moon shaped chloroplasts and complete loss of subcellular architecture within the diseased cells. While, in rhizobacterial mixture + chitosan treated leaves, the cells appeared somewhat closer to normal cells, with a small extent of distortion in chloroplasts. Our results imply that the rhizobacterial mixture + chitosan as the most efficient and alternative strategy for the management of insect transmitted ToLCV disease severity in tomato, besides promoting plant growth and fruit yield.

Key words: *Pseudomonas* spp., ToLCV, chitosan, biocontrol, *Bemisia tabaci*, transmission electron microscopy.

Tomato (*Solanum lycopersicon* L.) is an important and most widely grown vegetable crop of both tropics and sub tropics of the world and ranks second in importance among vegetables. But, there are many production constraints and it is affected by many diseases leading to substantial losses in yield. Besides fungal, bacterial and phytoplasmal infections, it is also affected by a large number of viral diseases (Anon, 1983), of which tomato leaf curl virus (ToLCV), is the most important viral pathogen in many parts of India (Saikia and Muniyappa, 1989; Harrison *et al*., 1991). Several approaches have been attempted to manage the tomato leaf curl virus. Chemical control measures create imbalances in the microbial community, which may be unfavorable to the activity of the beneficial organisms and may also lead to the development of resistant strains of pathogen. Developing resistant varieties can be difficult in the absence of dominant genes and development of new races of the pathogen overcoming host resistance.

Biocontrol by use of plant growth promoting rhizobacteria (PGPR) represents a potentially attractive alternative disease management approach since PGPR are known for growth promotion and disease reduction in crops (Jetiyanon and Kloepper, 2002) . A number of plant growth promoting rhizobacteria have been implicated in the biocontrol of virus diseases in

* To whom all correspondence should be addressed.

many crop plants such as tomato spotted wilt virus (Kandan *et al*., 2003), sunflower necrosis virus (Srinivasan *et al.,* 2005), banana bunchy top virus (Kavino *et al*., 2003) and TMV (Tobacco mosaic virus) in tomato (Kirankumar, 2007). However, application of a single biocontrol agent often results in inconsistent field performance as it is less likely to be active in different soil environment and agricultural ecosystems (Raupach and Kloepper, 1998) . Mixture of two or more biocontrol agents is likely to more closely mimic the natural situation and may, therefore, represent a more viable control strategy (De Boer *et al*., 1999).

Chitosan is one of the most studied elicitiors which trigger defensive mechanisms that constrain the invasion of pathogenic fungi, bacteria and viruses. It regulates the expression of resistance genes and induces jasmonate synthesis (Doares *et al*., 1995). Chitin and chitosan are naturally-occurring compounds that have potential role in the field of agriculture in controlling plant diseases and found to be active against viruses, bacteria and other pests (Abdelbasset *et al*., 2010). Therefore, we studied the biocontrol of ToLCV using rhizobacterial isolates either alone or in mixture along with chitosan formulations under field conditions.

MATERIALS AND METHODS

Rhizobacterial isolates

The rhizobacterial isolates were procured from the culture collection of Department of Biotechnology and Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad, India. The *Pseudomonas* isolates were maintained on nutrient agar medium. Based on results of the initial screening experiments, ten most effective PGPR strains were evaluated in a second experiment, and the three strains exhibiting the highest level of protection were tested in combination with chitosan under field conditions.

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 (University of Agricultural Sciences), Dharwad, during the crop season (Jan- June 2012). Five weeks old tomato seedlings, raised in a glasshouse were transplanted in the main field (plot size 20 m X 10 m) maintaining 75cm x 60cm spacing. In the chemical control treatment, confidor at the rate of 2 ml/L was sprayed at weekly intervals to control the vector, as per the package of practices for tomato crop.

PGPR and chitosan treatment

Seed treatment

Seeds of tomato were surface sterilized with one per cent sodium hypochlorite for 30 seconds rinsed in sterile distilled water and dried under sterile stream of air in a laminar air flow. Bacteria were grown in Nutrient broth medium on a shaker (150 rpm) for two days and centrifuged at 10,000 rpm for 5 min. The cell pellet was mixed with chitosan solution (5%). Surface sterilized tomato seeds were soaked in chitosan cell suspension and kept on a shaker at 28°C for 3 h till they became fully coated. Tomato seeds were coated with the bacterial pellet (*Pseudomonas* sp.) in chitosan solution resulting in densities of approximately 3 x 10^9 cfu/seed. The biocoated seeds were dried inside a laminar flow chamber and planted into pots with soil. Tomato seeds of the variety, Pusa Ruby (susceptible to ToLCV) were used in the experiment. All treatments were replicated five times and arranged in a randomized complete block design (RCBD).

Soil application

Erlenmeyer's flasks (100 ml) containing 50 ml nutrient broth were inoculated with a loopful of bacteria and incubated on a rotary shaker at 150 rpm for 3 days at 30° C. The broth was mixed with sterile lignite powder at 1:3 ratio and the formulation prepared. For soil application, the lignite based culture was applied to soil at the rate of 5kg/ha before sowing seeds and mixed well.

Foliar application

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. PGPR suspension treatments containing approximately 5×10^8 cfu/ml were sprayed on each plant. Control plants in pots without application of rhizobacteria were also maintained. 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.

ToLCV inoculation

The culture of ToLCV was obtained from the field in and around Agricultural College, UAS, Dharwad and inoculated to healthy tomato plants using whiteflies (*Bemisia tabaci)* as the vector and the plants maintained in the glass house throughout the period of study. Whiteflies were collected from cotton and tobacco plants in fields with the help of an aspirator by slowly turning the leaves upwards. Whiteflies were released on to the ToLCV diseased tomato plants grown in insect proof rearing cages and continuously maintained, thus making insects viruliferous.

Release of viruliferous insects

The viruliferous insects were collected 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 the 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.

Monitoring of the disease

Disease severity (% diseased plants severly affected) and the 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. Plants were examined daily for ToLCV symptoms (Severe curling and twisting, puckering, reduction in leaf size, reduced fruit formation and general stunting of the plant). The number of plants with severe symptoms were recorded. The percent disease severity was calculated using the formula,

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

Tomato leaf curl virus DNA extraction

One hundred mg of infected leaf tissue was ground in liquid nitrogen and the powder was transferred to 2 ml microfuge tubes containing 600 µl isolation buffer and both were mixed thoroughly and incubated at 65° C for 20 min. The DNA isolation buffer contained NaCI- 250mM, Tris Cl-200mM, EDTA- 25mM, SDS-0.5% .It was centrifuged at 13,200 rpm for 10 min at 4° C. The supernatant was transferred to two ml microfuge tubes and equal volume of phenol: chloroform was added. It was mixed properly and again centrifuged at 13,200 rpm for 10 min at 4°C. The upper aqueous layer was transferred to two ml microfuge tube and equal volume of chloroform: Isoamyl alcohol (24:1) was added and mixed properly. It was centrifuged at 13,200 rpm for 10 min at 4° C. The upper aqueous layer was transferred to 1.5 ml of microfuge tube and equal volume of chilled isopropanol was added. It was mixed properly and centrifuged at 13,200 rpm for 10 min.4^0C.The supernatant was discarded and the pellet washed in 100 µl 70% alcohol. The alcohol was evaporated at 37°C for 20 minutes and the pellet was dissolved in 50 µl $T_{10}E_1$ buffer.

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 (1X) and *Taq* polymerase (1U) were used. 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 gene of ToLCV. The PCR conditions consisted of an initial denaturation (94°C for 5 min) followed by cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min), extension (72°C for 1 min) and a final extension (72°C for 10 min). 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 with lambda DNA double digest DNA marker and amplified PCR products were visualized in ethidium bromide agarose gels under ultrav-violet light.

Transmission electron microscopy of ToLCV infected leaves

In order to study sub cellular changes in tomato leaves due to ToLCV, the healthy leaves, leaves treated by *Pseudomonas* sp.(206(4) +B-15+ JK-16) + chitosan and leaves of diseased plants were examined through transmission electron

microscopy (Spurr,1969). This was performed at RUSKA Lab, College of Veterinary Science, SVVU, Hyderabad, India.

Cleaning the surface of the specimen

The surface of 60 days old tomato leaves infected at 30 DAS by ToLCV were cleaned from contaminants by carefully rinsing them three times for 10 min in 0.1 M phosphate buffer (pH 7.2) at room temperature.

Fixation of the specimens

The samples were fixed in a 2.5 % glutaraldehyde solution (0.1 M phosphate buffer-pH- 7.2) and stored at 4^0 C for 24 h. All the samples were washed with 0.1 M phosphate buffer (pH 7.2) for three times. to remove the fixative from the interstitium, Secondary fixation was done with 2% aqueous osmium tetroxide prepared in 0.1 M phosphate buffer (pH 7.2) for 1h at room temperature. Then, the samples were dehydrated with graded ethanol (30 % to 100% - two changes) for 45 minutes each.

Infiltration of the specimens with a transitional solvent

The specimens were shifted to mixed solution of Araldite 6005 Resin and ethanol in the following manner: 1:2 (Araldite 6005 Resin: ethanol) for 1h followed by 2:1 (Araldite 6005 Resin: ethanol) for 1h followed by pure araldite 6005 resin for overnight.

Infiltration with resin and embedding the specimens

The samples were infiltrated and embedded in araldite 6005 resin and incubated for 72 h at 70-80^0C in a incubator.

Semi thin sections (1000 – 1200 nm) were made with a glass knife on ultra microtome (Lecia Ultra cut UCT-GA-D/E-1/00) mounted on a glass slide stained with toludene blue and observed under a light microscope for identification of specific area. Then ultra thin sections were (50-70 nm) made and mounted on copper grids and stained with saturated aqueous uranyl acetate and counter stained with Reynolds lead citrate. The stained samples were observed under Transmission electron microscope ("Hitachi", H-7500, Japan) at different magnifications and the photographs taken.

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 recorded 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 were subjected to the analysis of variance (ANOVA) using SPSS software version 17.0 and means were separated by Duncan's multiple range tests. Means were compared using the least significant difference (LSD) P d" 0.05.

RESULTS

Evaluation of the combination of rhizobacterial isolates and chitosan for the biocontrol of ToLCV in field conditions

Application of rhizobacteria along with chitosan significantly reduced ToLCV severity. At 75 days after inoculation (75 DAI) of the pathogen, the disease severity control varied from 83.60 to 93.30 per cent in combined treatment. Based on the initial screening, three isolates B-15, 206 (4) and JK-16 were selected for further characterization and biocontrol studies. Though all the treatments controlled the disease severity, the maximum disease severity reduction of 93.30 per cent was observed in tomato plants treated with *Pseudomonas* sp. 206(4) + B-15+ JK-16+ Chitosan

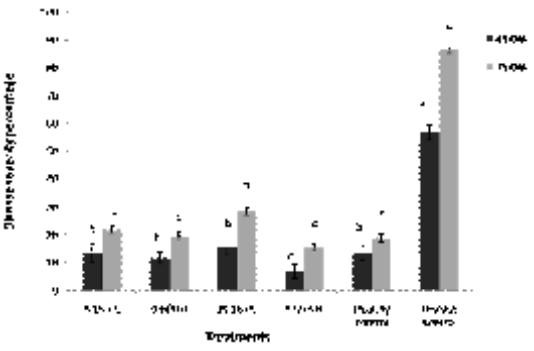

Fig. 1. Biocontrol of ToLCV disease by the various selected rhizobacteria in combination with chitosan . Disease severity percentage was observed at 45 and 75 days after innoculation (DAI) of viral pathogen. Different letters on bars indicate statistically significant between treated and control according to LSD ($P \leq 0.05$). Here, 1+2+3 =B-15+JK-16+206(4), C= Chitosan

at 75 DAI (Figure 1).

Subcellular changes in tomato leaves due to ToLCV and rhizobacterial inoculation

The cellular structures of the of the healthy, treated (*Pseudomonas* sp. 206(4) + B-15+ JK-16 + chitosan) and diseasd plants were studied by transmission electron microscopy (TEM), which showed the subcellular changes which occurred inside the cells due to ToLCV and rhizobacterial inoculation. Incase of diseased cells, a complete loss of subcellular architecture was seen. Cell boundaries were lost. Cells got completely shrunken with moon shaped chloroplasts, whereas

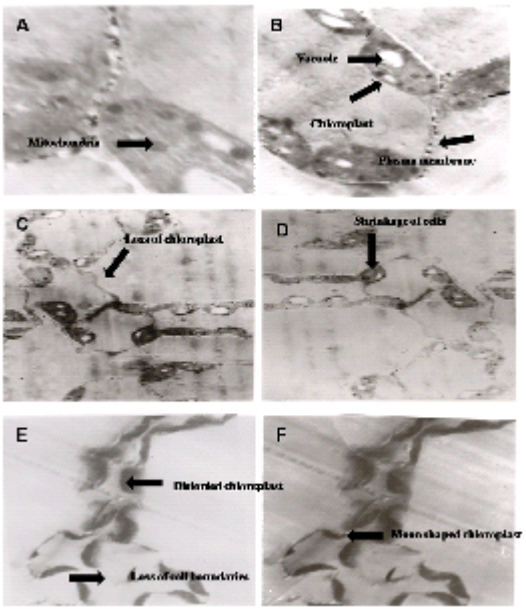

Fig. 2. TEM (Transmission Electron Microscopy) pictures depicting subcellular changes in tomato leaves due to ToLCV and rhizobacterial inoculation. Here, (A-B) : Healthy leaves; (C-D) : Virus infected leaves ,but inoculated with Pseudomonas sp.(206(4) +B-15+ JK-16) and chitosan; (E-F): Virus infected leaves

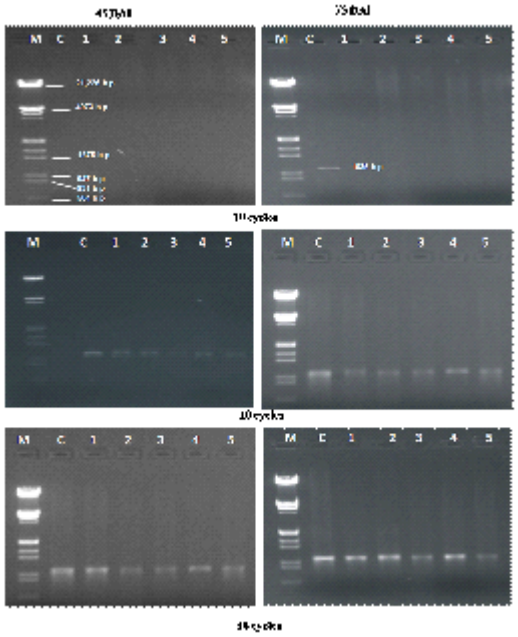

Fig. 3. Semi-quantitative PCR analysis of ToLCV in tomato. Here, Lane M : Lambda DNA double digest marker (*ECoR*™+*Hind* ™™™), Lane C: ToLCV amplicon after 10,20 and 30 cycles(control),Lane 1-5: PGPR treated plants with and without chitosan after 10,20 and 30 cycles; C. Disease control, 1. B-15+Chitosan, 2. 206 (4)+Chitosan, 3. B-15+206(4)+ JK-16+Chitosan, 4. JK-16+Chitosan, 5. Chemical control.The brightness intensity of band indicates the load of viral inoculum in treated and control plants. A slight reduction in the viral inoculum load was observed in all the rhizobacteria and chitosan treated plants. At each cycle, disease control plants exhibited highest viral inoculum load compared to treated plants

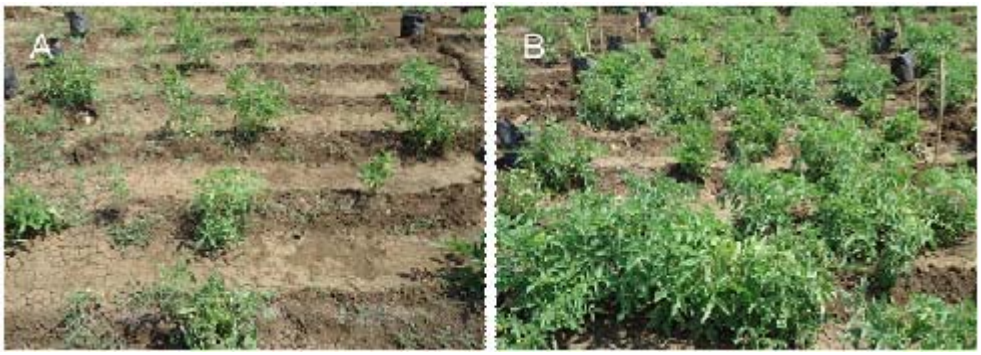

Fig. 4. General view of field experiment. Here, A : Uninoculated control (Disease control) and B: *Pseudomonas* sp.(206(4) + B-15+ JK-16) and chitosan treated

Table 1. Plant growth parameters of tomato as influenced by inoculation with rhizobacteria (at harvest) during Summer' 2012

Treatments	Plant height (cm)	Total biomass (g/plant)	Chlorophyll (SPAD)	Fruit yield (kg/plant)	Number of fruits per plant	Shelf Life (days)
Pseudomonas B-15+ Chitosan +ToLCV	35.0±1.14 b	38.18±0.717 c	30.8±0.769 b	0.721±0.019 c	20.2±0.663 c	7.4±0.244 b
Pseudomonas 206(4) + Chitosan +ToLCV	35.9±0.678 a,b	40.4±0.640 b	32.8±0.836 a,b	0.839±0.020 b	22.4±0.812 b	8.2±0.374 a,b
Pseudomonas JK-16+ Chitosan +ToLCV	34.7±0.653 b	34.48± 0.449 d	27.7±1.26 c	0.704±0.016 c	19.6±0.60 c	7.4±0.244 b
Pseudomonas (206(4) +B-15+JK-16) + Chitosan +ToLCV	38.1±1.60 a	42.09±0.520 a	35.6±1.34 a	1.011±0.032 a	26.8±1.06 a	8.6±0.246 a
Chemical control +ToLCV	30.2±0.930 c	29.56±0.438 e	26.0±0.715 c	0.568±0.013 d	17.4±0.509 d	5.6±0.249 c
Diseased control (only ToLCV)	26.02±0.396 d	23.8±0.474 f	18.4±0.884 d	0.310±0.019 e	9.4±0.508 e	5.2±0.200 c

Values are mean ± standard error (SE) of five replications per treatment observed at harvest. Means followed by the same letter are not significantly different from each other at P d" 0.05.

at some part of the cells, there was complete loss of chloroplast observed. No vacuolation was observed. But, incase of the treated leaves, cells appeared somewhat closer to normal cells. Some extent of distortion was seen in chloroplasts whereas other organells appeared to be normal. Shrikage of protoplast was observed. Cell membrane was intact. At intercellular junction, electron densed materials were seen. Vacuolation was observed inside the chloroplast. Healthy plant cells appeared normal with intact histological structure (Figure 2).

Detection of ToLCV inoculum in rhizobacteria and chitosan treated leaves

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 +chitosan-treated and untreated tomato plants were tested for the presence of the virus. The amplicon size obtained after 10, 20 and 30 cycles was 1035 base pair which was compared with lambda DNA Double digest DNA marker.The intensity of amplicon was found too less in case of plant sample treated with rhizobacterial mixture and chitosan. At both 45 DAI and 75 DAI, *Pseudomonas* sp. (206(4) + B-15+ JK-16) and chitosan treated plants showed the least viral load (Figure 3). However, this needs to be further validated.

Effect of inoculation of rhizobacteria along with chitosan on growth promotion and yield of tomato plants

Inoculation of *Pseudomonas* sp. 206(4) + B-15+ JK-16+ Chitosan significantly improved growth components of tomato, when compared to the remaining rhizobacterial isolates (Figure 4). It increased the plant height by 31.70 percent, biomass by 43.45 percent, chlorophyll content by over 48.31 percent, number of fruits per plant and yield by 64.92 and 69.33 percent respectively compared to the viral pathogen inoculated control (Table 1). The size, quality and shelf life of fruits were improved due to rhizobacterial and chitosan treatment (Figure 5).The shelf life due to *Pseudomonas* sp. 206(4) + B-15+ JK-16+ Chitosan treatment was extended by 39.53 percent.

Fig. 5. Effect of inoculation of rhizobacterial mixture and chitosan on fruit quality of tomato. Here, A : Uninoculated control (Disease control) and B: *Pseudomonas* sp.(206(4) + B-15+ JK-16) and chitosan treated

DISCUSSION

Insect transmitted viruses are known to be the most lethal agents affecting the yield, frequently encountered by the farmers. Effective control of insect borne viral diseases is problematic since most viruses are transmitted by highly mobile insects and may colonize fields rapidly, before their presence is felt. Therefore, an attempt was made to evaluate the biocontrol ability of rhizobacterial isolates under high virus-vectors pressure conditions. Intriguingly, supplementation of rhizobacteria along with chitosan significantly impaired the severity of ToLCV. Earlier studies conducted by 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. Similarly, Postma *et al.* (2009) observed the control of *Pythium aphanidermatum* in cucumber with a combined treatment of *Lysobacter enzymogenes* strain 3.1T8 along with chitosan resulted in the reduction in number of diseased plants approximately by 50-100 per cent. Murphy *et al*. (2003), observed significant protection augumented by PGPR when combined with chitosan against CMV (cucumber mosaic virus) in tomato plants.. Wisniewska-Wrona *et al*. (2007) reported that the oligomers and partially degraded products of chitosan totally retarded (100 per cent) the alfalfa mosaic virus (AMV) and and in addition, inhibited (32 per cent) the growth of resistant tobacco mosaic virus (TMV). 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 fruit. Similar observations were reported with the potato virus X (PVX), tobacco mosaic and necrosis viruses, alfalfa mosaic virus, peanut stunt virus, and cucumber mosaic virus (Pospieszny *et al.*, 1991; Chirkov *et al*., 2002). On the bean leaves, local infections produced by alfalfa mosaic virus (AMV) were completely controlled with the highest chitosan concentration (0.1%) either sprayed or added to the inoculum (Pospiezny *et al*., 1991). Similar kind of inhibition was reported on the tomato leaves treated with chitosan at the same concentration and inoculated with potato spindle tuber viroid **(**Muniyappa and Veeresh,1984). In all these studies, systemic resistance was induced by chitosan on various host virus combinations. Therefore, scientific evidences suggests that chitosan treatments along with PGPR significantly reduces the virus infections in various plants.

The complete loss of subcellular architecture within the diseased cells and the appearance of moon shaped chloroplast were explained by Manners and Scott (1985). They observed that one of the earliest alternations in leaf tissue due to pathogenic infection was the selective breakdown of chloroplast polysomes. The reduced chlorophyll content in diseased control treatment could also be attributed due to the breakdown of chlorophyll pigments by the pathogen and inhibition of chloroplast development by the pathogen as reported by various workers (Robert and Wood, 1982; Kirankumar, 2007; Patil, 2010). They also observed reduced chlorophyll content in the leaves of tomato plants challenge inoculated with TMV and bhendi plants inoculated with BYVMV.

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 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 as determined by ELISA method. Malathi *et al.* (2011) also detected Rice tungro virus particles from infected rice leaves by RT- PCR based diagnosis. In addition to suppressing the viral

disease, *Pseudomonas* sp. 206(4) + B-15+ JK-16+ Chitosan greatly improved plant growth, biomass, chlorophyll content and yield. 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). Scientific studies by Manjula and Podile (2005) suggests that 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 comparison with an increase of 21 and 30 per cent, respectively by *Bacillus subtilis* AF1alone. Our results have shown that the combined treatment of mixture of PGPR and chitosan also enhanced the tomato fruits quality and shelf-life. The extended shelf life may be due to reduction of polygalacturonase (PG) activity which resulted in increased pericarp tissue firmness and increased resistance to fruit pathogen damage (Kramer *et al*., 1992).

Thus, the study has brought out the biocontrol potential of the selected combination of PGPR with chitin supplemented formulations. Chitin enhanced the efficacy of PGPR in reducing the disease severity of ToLCV as well as in promotion of tomato growth and yield either through induced antagonistic gene expression or chitosan serving as C –and N-source for the antagonists or both. These findings suggest that the effects of PGPR along with chitosan on ToLCV in tomato may be associated with the direct antiviral property against the pathogens.

ACKNOWLEDGMENTS

The first author acknowledges the financial assistance received from University of Agricultural Sciences, Dharwad, India in the form of University Merit Scholarship during the course of study. I convey my sincere thanks to Dr. M. Lakshman, Professor and officer-in-charge, RUSKA LABS, College of Veterinary Science, Hyderabad for their valuable help during my transmission electron microscopy studies.

REFERENCES

1. Abdelbasset EH, Adam LR, Hadrami IE, Daayf F, Chitosan in Plant Protection. *Marine Drugs*, 2010; **8**: 968-987.
2. Anonymous, Pest control in Tropical Tomatoes. Centre for overseas pest research. London, 130 1983.
3. Benhamou N, Lafontaine PJ, Nicole M, Induction of systemic resistance to Fusarium crown and root rot in tomato plants by seed treatment with chitosan. *Phytopathol*, 1994; **84**: 1432–1444.
4. Chirkov SN, The antiviral activity of chitosan. *Appl Biochem Microbiol*, 2002; **3**: 81–89.
5. De-Boer M, van der Sluis I, van Loon LC, Bakker PAHM, *Combining fluorescent Pseudomonas spp. strains to enhance suppression of Fusarium wilt of radish. Eur Journal Plant Pathol*, 1999; **105**: 201–*210*.
6. Doares S H, Syrovets T, Weiler EW, Yan CA, Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. Proc National Academy Science, USA, 1995; **92**: 4095-4098.
7. Earnapalli VN, Screening of antagonistic microorganisms for biological control of early blight of tomato caused by Alternaria solani. Dharwad, India, 115p. (M. Sc Agri. Dissertation, University of Agricultural sciences, Dharwad) 2005.
8. Harrison BD, Muniyappa V, Swanson MM, Roberts IM, Robinson DJ, Recognition and differentiation of seven whitefly transmitted Gemini viruses from India and their relationships to African cassava mosaic and Thailand mungbean yellow mosaic viruses. *Annals App Biol* , 1991; **118**: 299-308.
9. Jetiyanon K, Kloepper JW, Mixtures of plant growth promoting rhizobacteria for induction of systemic resistance against multiple plant diseases. *Biol Control*, 2002; **24**: 285-291.
10. Kandan A, Radjacommare R, Ramiah M, Ramanathan A, Samiyappan, R, PGPR induced systemic resistance in cowpea against tomato spotted wilt virus by activating defense against tomato spotted wilt virus by activating defense related enzymes and compound. In: Sarma, Y.R.(eds). Proceedings of the Sixth International Workshop on Plant Growth Promoting Rhizobacteria. IISR Publishers, Calicut, India, 2003; 480-486.
11. Kavino M, Harish S, Kumar N, Soorianathasundaram K, Ramanathan A, Samiyappan R, PGPR Induced systemic resistance against banana bunchy top virus in banana. *In*: Sarma, Y.R.(eds). Proceedings of the Sixth International Workshop on Plant Growth Promoting Rhizobacteria. IISR Publishers, Calicut, India, 2003; 486-492.

12. Kim YC, Jung H, Kim KY, Park SK, 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**: 373–382.
13. Kirankumar R, Evaluation of Plant growth promoting rhizobacterial stains against TMV on tomato. Dharwad, India, 120p. (M. Sc Agri. Dissertation , University of Agricultural sciences, Dharwad) 2007.
14. Kramer MR, Sanders HB, Waters C, Sheehey RE, Hiatt WR, Post-harvest evaluation of transgenic tomatoes with reduced levels of polygalacturonase: processing, firmness and disease resistance. *Postharvest Biol Technol,* 1992; **1**: 241-255.
15. Liana LC, Liyan XB, Luping ZB, Qiying LB, Induction of systemic resistance in tobacco against Tobacco mosaic virus by *Bacillus* spp. *Biocontrol Sci and Technol,* 2011; **21**: 281-292.
16. Malathi P, Umarani B, Jarati S, Krishnaveni D, Balachandran SM, Neeraja CN, Laha GS, Viraktamath BC, Satendra KM, PCR based diagnosis of rice tungro bacilliform and spherical virus infected plants and insect vectors. *Indian J Plant Prot*, 2011; **4**: 294-298.
17. Manjula K, Podile AR, Increase in seedling emergence and dry weight of pigeon pea in the field with chitin supplemented formulations of *Bacillus subtilis* AF1. *World J Microbiol Biotechnol,* 2005; **21**:1057-1062.
18. Manners JM Scott K J, Reduced translatable messenger RNA activities in leaves of barley infected with *Erysiphae graminis*. *Physiol Plant Pathol* **,** 1985; **26**: 297-308.
19. Muniyappa V, Swanson MM, Duncan GH, Harrison BD, Particle purification, properties and epitope variability of Indian tomato leaf curl geminivirus. *Annals App Bio,* 1991; **118**: 595-604.
20. Muniyappa V, Veeresh GK., Plant virus diseases transmitted by whiteflies in Karnataka. Proceedings of Indian Academy of Sciences 1984; **93**: 397-406.
21. Murphy JF, Reddy MS, Ryu CM, Kloepper JW, Li R., Rhizobacteria mediated growth promotion of tomato leads to protection against *Cucumber mosaic virus*. *Virol* 2003; **93**(10):1301-1307.
22. Patil MR., Potential of rhizobacteria to control bhendi yellow vein mosaic virus in bhendi. Dharwad, India, 115p. (M. Sc Agri. Dissertation, University of Agricultural sciences, Dharwad), 2010.
23. Pospieszny H., Antiviroid activity of chitosan. *Crop Prot* 1997; **16**: 105–106.
24. Pospieszny H, Chirkov S, Atabekov J., Induction of antiviral resistance in plants by chitosan. *Plant Sci* 1991; **79**: 63–68.
25. Postma J, Stevens LH, Wiegers GL, Davelaar E, Nijhuis EH., Biological control of *Pythium aphanidermatum* in cucumber with a combined application of *Lysobacter enzymogenes* strain 3.1T8 and chitosan. *Biol Control* 2009; **48**: 301–309.
26. Punja ZK, Yip R., Biological control of damping-off and root rot caused by *Pythium aphanidermatum* on greenhouse cucumbers. *Can J Plant Pathol* 2003; **25**: 411–417.
27. Raupach GS, Kloepper JW., Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. *Phytopathol* 1998; **88**: 1158–1164.
28. Robert PL, Wood KR., Effects of a severe (P6) and a mild (W) strain of cucumber mosaic virus on tobacco leaf chlorophyll, starch and cell ultrastructure. *Physiol Plant Pathol* 1982; **21**: 31-37.
29. Saikia AK, Muniyappa V., Epidemiology and control of tomato leaf curl virus in *Southern India. Trop Agric* 1989; **66**: 350–354.
30. Spurr AR., Electron microscopy techniques. *J Ultrastructural Res* 1969; **26**: 311-316.
31. Srinivasan K, Surendiran G, Maathivanan N., Pathological and molecular biological investigations on sunflower necrosis virus (SNV) and ISR mediated biological control of SNV by PGPR strains. Asian Conference on Emerging Trends in Plant- Microbe Interaction, Chennai, India, 2005; 152.
32. Vasanthi VJ, 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): 1463–1472.
33. Wisniewska-Wrona M, Niekraszewicz A, Ciechanska D, Pospieszny H, Orlikowski LB., Biological properties of chitosan degradation products. Polish Chitin Society, Monograph , 2007; 149-156.
34. Yu T, Hong YL, Xiao DZ., Synergistic effect of chitosan and *Cryptococcus laurentii* on inhibition of *Penicillium expansum* infections. *Int J Food Microbiol* 2007; **114**: 261–266.
35. Zehnder GW, Yao C, Murphy JF, Sikora ER, Kloepper W., Induction of resistance in tomato against cucumber mosaic cucumovirus by plant growth promoting rhizobacteria. *Biocontrol* 2000; **45**: 127-137.