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Histological and histochemical changes induced by the amendment of chitosan and inoculation of *Pseudomonas* sp. against Tomato leaf curl virus (ToLCV) in tomato (*Lycopersicon esculentum* Mill.)

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This study was conducted to evaluate the comparison between the histological and histochemical changes in leaves of tomato due to tomato leaf curl virus (ToLCV) with or without plant growth-promoting rhizobacteria (PGPR) and chitosan treatments. The combined treatment of chitosan and *Pseudomonas* sp. induced a significant ($P=0.05$) increase in the activities of polyphenoloxidase (PPO), peroxidase (POD), chitinase, phenylammonialyase (PAL) and enhanced the content of phenolic compounds in tomato leaves. Furthermore, the combined application reduced the disease severity (%) of ToLCV in tomato plants. Although, the histopathological and histochemical studies revealed more destruction in the diseased plants, these changes could be reduced to the level of the healthy plants due to combined treatment of chitosan and *Pseudomonas* sp.

Key words: *Pseudomonas* sp., Tomato leaf curl virus (ToLCV), induced systemic resistance, chitosan, biocontrol, *Bemisia tabaci*, histopathology.

INTRODUCTION

Tomato (*Solanum lycopersicon* L.) is one of the important and most widely grown vegetable crops of both tropics and sub tropics in the world and ranks second in importance among vegetables. Recently, there has been more emphasis on tomato production not only as a source of vitamins, but also as a source of income and food security in many countries including India. There are many production constraints and it is affected by several diseases leading to substantial losses in yield. Of all the

viral diseases reported on tomato, tomato leaf curl virus *Tomato leaf curl virus* (ToLCV), a Geminivirus (family *Geminiviridae*, genus *Begomovirus*) is the most important and destructive viral pathogen in many parts of India (Saikia and Muniyappa, 1989; Harrison et al., 1991). The disease is transmitted by whitefly (*Bemisia tabaci*) (Muniyappa and Veeresh, 1984).

The investigation of plant response to elicitors is one of the most rapidly developing lines of inquiry in plant

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physiology.

The elicitors stimulate the contact between plants and phytopathogens and, thereby, trigger defensive mechanisms that constrain the invasion of pathogenic fungi, bacteria and viruses. Chitosan is one of the most studied elicitors, and it regulates the expression of resistance genes and induces jasmonate synthesis (Doares et al., 1995). These molecules have been shown to display toxicity and inhibit fungal growth and development. They were reported to be active against viruses, bacteria, and pests as well (Abdelbasset et al., 2010). Based on these properties 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.

Histopathological and biochemical changes occur in tomato leaves after infection by ToLCV resulting in external manifestation in the form of different symptoms. The plants inoculated with *Tobacco mosaic virus* (TMV) in tomato exhibited the disintegration of palisade tissue with wider space in spongy parenchyma (Alok et al., 1986). There was reduction in the amount of reducing sugars, non-reducing sugars, total sugars, starch and total chlorophyll in the plants infected with yellow mosaic virus as compared to healthy plants (Thind et al., 1996). In case of papaya leaves infected with the *Papaya ring spot virus* (PRSV), the palisade layers and spongy cells became smaller, and the leaves have been shown with broad inter cellular spaces (Singh, 1971).

Biocontrol using plant growth-promoting rhizobacteria (PGPR) agent 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 viral diseases in 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 in tomato (Kirankumar, 2007). These viruses have been controlled essentially through induced systemic resistance (ISR) by activating defense genes encoding chitinase, beta-1, 3 glucanase, peroxidase, phenylammonia lyase and other enzymes (Srinivasan et al., 2005; Kirankumar, 2007) and chemicals (M'piga et al., 1997). Several antagonistic microorganisms such as *Pseudomonas*, *Bacillus*, *Streptomyces*, *Gliocladium* and *Trichoderma* spp. have the potential to control a variety of crop diseases. In other studies, beneficial microorganisms were combined with a natural compound such as chitin or chitosan to improve their biocontrol efficacy (Sid-Ahmed et al., 2003). The aim of the present study was to compare the histochemical changes in leaves of tomato due to ToLCV, with or without PGPR amendments along with chitosan treatments; and to study the elicitation of Induced systemic resistance (ISR) molecules the defense responses of plants induced by them.

MATERIALS AND METHODS

Collection and preparation of viral inoculums

The culture of ToLCV was obtained from the virus infected tomato plants in the field, Agricultural College, UAS, Dharwad, India, and inoculated to healthy tomato plants using whiteflies (*Bemisia tabaci*) as the vector and the infected plants were maintained in the glasshouse throughout the period of study.

Vector culture rearing

Whiteflies were collected from cotton and tobacco plants in the fields with the help of an aspirator by turning the leaves slightly upwards. Whiteflies were released onto the ToLCV diseased tomato plants grown in insect proof rearing cages which make the insects viruliferous after sucking of whiteflies on diseased leaves and their population were maintained by introducing younger tomato plants into the rearing cages.

Release of viruliferous insects

The viruliferous insects were collected from the diseased plants with the help of an aspirator, and released onto the leaves of healthy PGPR treated tomato seedlings. Immediately, the seedlings were placed in an insect proof rearing cage and insects allowed to feed on them for a week to cause 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.

Field study

A field experiment was conducted to assess the effect of the selected PGPR strain and chitosan on reduction of disease severity and growth promotion in tomato plants. It was carried out at main agricultural research station, UAS, Dharwad, India, during summer (Jan- May, 2012). Five-week-old seedlings of variety pusa ruby, raised in a glasshouse were transplanted in the main field with plot size 20 m × 10 m and 75 cm × 60 cm spacing.

Rhizobacterial treatment

The bacteria were cultured on nutrient broth (Peptone, 5 g; Beef extract, 3 g; NaCl, 5 g; pH 6.8-7.2/Lit) and incubated at 28°C for 48 h with shaking at 150 rpm. Bacteria were pelleted after centrifugation for 5 min at 10000 × g, and the pellet was mixed with sterile 1% carboxy methyl cellulose (CMC) suspensions (Himedia, Mumbai, India). The seeds were surface sterilized with sodium hypochlorite solution, placed in CMC-cell suspensions, air dried inside a laminar flow chamber and the bio-coated seeds were sown in the plastic pots (10" diameter) having 10 kg capacity of soil. For soil application, the lignite based culture (1:3) was applied to soil @ 5 kg/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 and 20 days after sowing (DAS). Control plants in pots without application of rhizobacteria were also maintained. All treatments were replicated five times and arranged in a randomized complete block design (RCBD).

Chitosan treatment

Bacteria were grown in nutrient broth medium on a shaker (150 rpm) at 28°C for two days and centrifuged at 10000 × g 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%). Crab-shell chitosans (deacetylation of about 90%) from Sigma-Aldrich were used in this work. The surface sterilized tomato seeds were soaked in chitosan-cell suspensions and kept on a shaker for 3 h at 28°C and 100 rpm. The seeds were shaken in chitosan solution until they became fully coated. The biocoated seeds were dried inside a laminar flow chamber. 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 the pH was adjusted to 6.5 with 1 N NaOH.

Sample collection, Enzyme and phenol estimation

Leaf samples were collected at 45 DAS and 75 DAS from both inoculated and uninoculated (control) tomato plants. They were frozen immediately in liquid nitrogen, ground to a powder and stored at -80°C until determination of phenylammonia lyase, chitinase, polyphenol oxidase and peroxidase activities. The peroxidase activity was assayed spectrophotometrically following the method described by Mahadevan and Sridhar (1986). The phenylammonia lyase 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 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).

Preparation of samples for histochemical changes using various staining techniques

Affixing, deparaffinising and hydrating the sections onto the slides

PGPR treated plants with or without amendment of chitosan and diseased plants were taken for the histochemical analysis. Tomato leaves after 45 days after inoculation (DAI) were used for histopathological studies. Leaf bits (1.0 × 0.5 cm) were processed following the paper boat technique (Jensen, 1962). The sections of 10 µm size embedded samples were taken using Leica microtome. An adhesive was prepared using gelatin at 1.5 g/100 ml distilled water, and a little quantity of potassium dichromate added to prevent fungal growth. A few drops of gelatin were added on to the surface of clean microslide. Sections were carefully placed on adhesive and slides were warmed slightly over hot plate maintained at 50°C for 1-2 min to facilitate flattening and stretching of section ribbons. The excess adhesive was drained off and the slides were dried for 24 h at room temperature. The sections were deparaffinised using xylene and were then treated with different grades of alcohol for gradual dehydration. Later, the sections were subjected to staining either directly or after hydration depending on the requirement. After each step, the slides were blot dried to remove excess chemical adhered to the slides.

Staining, dehydration and mounting the sections for various biochemical analyses

The sections were subjected to histological and histochemical staining for localization of different cellular chemical compounds namely insoluble polysaccharides, proteins and nucleic acids. To observe anatomical changes in diseased leaves, sections were passed through safranin and fast green stains. Sections were

stained in 1% safranin (1 g of safranin in 100 ml of 50% alcohol) for 2 h and then dehydrated in 50, 70 and 90% alcohol for 5 min, stained with 0.5% fast green (0.5 g of fast green in 100 ml of 95% alcohol) for 5 min and again dehydrated with 95% alcohol for 5 min, cleaned in xylene and mounted in DPX. The assessment of total insoluble polysaccharides was followed by Periodic acid Schiff's (PAS) method (Hatchkiss, 1948). The reagents were prepared as described by Longley (1952). For the assessment of total proteins, the method developed by O' Brien and Mc Cully (1981) was used. For the staining of nucleic acids, the method of Toluidine Blue O was used (Chayen et al., 1973). The dye Toluidine blue was used for the purpose of detecting the richness of RNA and DNA in cells. The slides were photographed using a Axiostar plus ("Carl Zeiss") Bright field microscope with Canon power shot G2 digital camera attachment.

Statistical analysis

The data obtained from field experiments were subjected to Randomized Complete Block Design analysis (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

Structural staining for anatomical changes

Histopathological studies were conducted to study the changes occurring due to ToLCV infection as well as to study to what extent these changes are reduced by inoculation with biocontrol agents. The histopathological studies revealed more anatomical destruction in leaves of diseased plants as compared to treated and healthy plants. There were more intercellular spaces. The palisade cells were markedly reduced in size, and lost their columnar compact nature (Figure 1). The reduction in size of leaf tissues and their destruction may be due to metabolic changes in tissues causing hypotrophy in palisade and spongy parenchyma cells. Similar observations were recorded in infected papaya leaves (Singh, 1971), *Cucumber mosaic virus* (CMV) affected chilli leaves (Mishra and Singh, 1973), CMV infected tobacco leaves (Ehara and Mishawa, 1975), TMV infected tomato (Alok et al., 1986) and in pigeonpea infected by sterility mosaic virus (Singh and Rath, 1996a).

Effect on polysaccharides content in leaves

The studies also revealed reduction in insoluble polysaccharides in palisade and spongy parenchyma cells of diseased plants as compared to healthy plants, which showed rich concentration of polysaccharides in the cells. The treatments receiving *Pseudomonas* sp. 206(4) +chitosan and *Pseudomonas* sp. B-15+Chitosan showed almost the same concentration of polysaccharides as seen in healthy cells whereas, *Pseudomonas* sp. 206(4) and *Pseudomonas* sp. B-15 showed a slight reduction in

Figure 1. Structural changes in tomato leaves after treatment with various selected rhizobacteria in combination with or without chitosan. The leaves were sampled 45 DAI for dissection.

Figure 2. Histochemical changes (polysaccharides) in tomato leaves after treatment with various selected rhizobacteria in combination with or without chitosan. The leaves were sampled 45 DAI for dissection.

concentration of polysaccharides in palisade and spongy parenchyma cells (Table 1 and Figure 2). The reduction

in polysaccharides may be due to varied metabolism in infected tissues. Several workers have reported signifi-

Table 1. Histochemical changes in healthy, treated and ToLCV infected leaves of tomato plants (At 45 DA).

Status	Histochemical	Different regions of leaf		
		Epidermis	Palisade parenchyma	Spongy parenchyma
Healthy	Polysaccharides	+++	+++	+++
	Proteins	++++	++++	+++
	Nucleic acids	++	++	++
Diseased	Polysaccharides	+	+	+
	Proteins	+	+	+
	Nucleic acids	++++	++++	++++
<i>Pseudomonas</i> sp.206(4)+Chitosan treatment	Polysaccharides	+++	+++	+++
	Proteins	+++	+++	++
	Nucleic acids	++	++	++
<i>Pseudomonas</i> sp.206(4) treatment	Polysaccharides	++	++	++
	Proteins	++	++	++
	Nucleic acids	+++	+++	+++
<i>Pseudomonas</i> sp.B-15+Chitosan treatment	Polysaccharides	+++	+++	+++
	Proteins	+++	+++	++
	Nucleic acids	++	++	++
<i>Pseudomonas</i> sp.B-15 treatment	Polysaccharides	+	+	+
	Proteins	++	++	++
	Nucleic acids	+++	+++	+++

++++, Very rich; +++ rich; ++ Medium; +, Low.

cant decrease in total sugars, reducing sugars and non-reducing sugars in diseased leaves (Khurana, 1970; Singh, 1973; Johri, 1975).

Effect on nucleic acid content in leaves

Nucleic acid content was found to be greater in palisade and spongy cells of diseased leaves when compared to healthy leaves. The manifestation of increase in nucleic acid content might be due to the combined effect of host and viral nucleic acid. Usually, concentration of virus in infected cells increases rapidly upon infection, thus, increasing their nucleic acid concentration in such cells. The treatments of *Pseudomonas* sp. 206(4)+ chitosan, and *Pseudomonas* sp. B-15 + chitosan showed almost similar nucleic acid quantity as seen in healthy cells whereas, *Pseudomonas* sp. 206(4) and *Pseudomonas* sp. B-15 showed a slight increase in nucleic acid content in palisade and spongy parenchyma cells (Table 1 and Figure 3). Our results are corroborative with the results obtained by Joshi and Dubey (1974) for the CMV infected chili-pepper leaves, infected nuclei of tobacco (Russo and Martell, 1975), nucleic acids and chloroplasts of

tomato cells infected by ToLCV (Channarayappa et al., 1992). Similar effect was also reported by Singh and Rath (1996b), where the presence of foreign ribonucleo protein in cytoplasm and nucleus of virus infected pigeonpea and increase in total nucleic acid in virus infected host tissues of papaya (Johri, 1975).

Effect on protein content in leaves

There was a significant higher reduction of protein content in palisade and spongy parenchyma cells from infected plants when compared to healthy plants; this might be due to the degradation of host protein or reduction in synthesis of protein subsequent to viral infection since virus uses host cell contents for replication. The combined treatment of the plants with *Pseudomonas* sp. 206(4)+chitosan, *Pseudomonas* sp. B-15+chitosan showed almost the same quantity of proteins as seen in healthy cells; while, the individual application of *Pseudomonas* sp. 206(4) and *Pseudomonas* sp. B-15 showed a reduction in protein content in palisade and spongy parenchyma cells (Table 1 and Figure 4). The reduction of protein content by 41% in diseased papaya

Figure 3. Histochemical changes (nucleic acid) in tomato leaves after treatment with various selected rhizobacteria in combination with or without chitosan. The leaves were sampled 45 DAI for dissection.

Figure 4. Histopathological changes (proteins) in tomato leaves after treatment with various selected rhizobacteria in combination with or without chitosan. The leaves were sampled 45 DAI for dissection.

Figure 5a. Effect of treatment with various selected rhizobacteria in combination with or without chitosan on phenol content in tomato leaves. Phenol content was estimated 45 days after sowing (DAS). Different letters on bars indicate statistically significant between treated and control according to LSD ($P=0.05$). Each value represents the mean of three replications per treatment. Error bars indicate $\pm$ standard error (SE). Here, C represents chitosan.

fruits was earlier reported (Singh et al., 1977; Sun, 1985). The histochemical analysis from our study revealed that there was an increase of nucleic acid and decrease of insoluble polysaccharides, and also protein content due to ToLCV infection.

Effect of rhizobacteria and chitosan on disease resistance against ToLCV through induced systemic resistance in tomato plants

Bio-control agents, mainly bacterial inoculants are believed to induce systemic defense responses in the plants besides other antagonistic mechanisms. Induction of defense responses by plant-growth-promoting rhizobacteria (PGPR) is largely associated with the production of pathogenesis related (PR) proteins like β -1,3-glucanase and the defense enzyme phenylalanine ammonia-lyase and oxidative enzymes like peroxidase and polyphenol oxidase (Compant et al., 2005). There was a greater level of phenolics in plants inoculated with *Pseudomonas* sp. and chitosan compared to uninoculated plants. These results are in agreement with previous results, where there was increased phenolics content in cowpea due to *P. fluorescens* inoculation which, in turn, protected plants from spotted wilt virus (Kandan et al., 2003). An increased chitinase activity resulted in preventing the damage caused by viral pathogen and, thus, increased the disease control percentage in all the rhizobacteria treated plants. There was induction in biosynthesis of defense molecules in all the treated plants. Combined application of *Pseudomonas*

sp. 206(4) and chitosan on tomato plants resulted in the highest synthesis of phenol content, PO, PPO, chitinase and PALase activity which accounts to 30.55, 49.31, 38.79, 32.90 and 34.78% respectively; higher than the diseased control. The role of PPO in disease resistance is to oxidize phenolic compounds to quinones, which are often more toxic to microorganisms than the original phenols and the enzyme itself is inhibitory to viruses by inactivating the RNA of the virus (Vidhyasekaran, 1988). Though all the treatments induced biosynthesis of phenolics, PAL, peroxidase, chitinase and polyphenol oxidase, the *Pseudomonas* sp. 206(4) + Chitosan treatment showed a greater defense activity compared to disease control (Figure 5). Cinnamic acid, the product of PAL, is directly linked to cell lignification processes and the highest levels of PAL activity usually occur about one day after initial infection of pathogen (Podile and Laxmi, 1998). The PO and PPO activities are linked to lignification and generation of hydrogen peroxides at later stages of infection, which inhibit pathogens directly, or generation of other free radicals with antimicrobial activity, that restrict the development of pathogens (Silva et al., 2004).

Application of rhizobacteria in combination with or without chitosan reduced the ToLCV severity by 58-70%. The treatment receiving *Pseudomonas* sp. 206(4)+chitosan reduced the disease severity by 68% whereas the treatment receiving *Pseudomonas* sp. B-15+chitosan exhibited 68.30% ToLCV severity control under field condition (Figure 5f). Thus, the observed induction of enzymes and the corresponding reduction in ToLCV infection in tomato supports the hypothesis that the

Figure 5b. Effect of treatment with various selected rhizobacteria in combination with or without chitosan on phenylalanine ammonia lyase activity in tomato leaves. Phenylalanine ammonia lyase activity was estimated 45 days after sowing (DAS). Different letters on bars indicate statistically significant between treated and control according to LSD ($P=0.05$). Each value represents the mean of three replications per treatment. Error bars indicate $\pm$ standard error (SE). Here, C represents chitosan.

Figure 5c. Effect of treatment with various selected rhizobacteria in combination with or without chitosan on peroxidase activity in tomato leaves. Peroxidase activity was estimated 45 days after sowing (DAS). Different letters on bars indicate statistical significance between treated and control according to LSD ($P=0.05$). Each value represents the mean of three replications per treatment. Error bars indicate $\pm$ standard error (SE). Here, C represents chitosan.

resistance induced by isolates is systemic.

The increase in callose content as well as induction in ribonuclease and β -1,3 glucanase in potato plants as defense response against potato virus X (PVX) were observed when plants were sprayed with chitosan solution (1 mg/ml). In other observation, there was

enhanced activation of catalase, PAL, Peroxidase, PPO and chitinase levels in sunflower when seeds were treated with chitosan (5%) for controlling downy mildew (Chirkov et al., 2001; Nandeeshkumar et al., 2008). Similar observations were reported with the potato virus X, tobacco mosaic and necrosis viruses, alfalfa mosaic

Figure 5d. Effect of treatment with various selected rhizobacteria in combination with or without chitosan on chitinase activity in tomato leaves. Chitinase activity was estimated 45 days after sowing (DAS). Different letters on bars indicate statistically significant between treated and control according to LSD ($P=0.05$). Each value represents the mean of three replications per treatment. Error bars indicate $\pm$ standard error (SE). Here, C represents chitosan.

Figure 5e. Effect of treatment with various selected rhizobacteria in combination with or without chitosan on polyphenol oxidase activity in tomato leaves. Polyphenol oxidase activity was estimated 45 days after sowing (DAS). Different letters on bars indicate statistical significance between treated and control according to LSD ($P=0.05$). Each value represents the mean of three replications per treatment. Error bars indicate $\pm$ standard error (SE). Here, C represents chitosan.

virus, cucumber mosaic virus (Chirkov, 2002).

Conclusion

In the present research, we showed that *Pseudomonas* sp. 206(4) combined with chitosan is a potential

biocontrol agent with strong and reproducible biocontrol effects on controlling disease severity of ToLCV in tomato plants. Histochemical studies revealed reduction in insoluble polysaccharides and proteins and increase in nucleic acid content in palisade and spongy parenchyma cells of diseased plant as compared to treated and healthy plant. Histochemical studies also clearly indicated

Figure 5f. Disease severity percentage exhibited by selected rhizobacteria in combination with or without chitosan after 75 days of pathogen (ToLCV) inoculation. Different letters on bars indicate statistical significance between treated and control according to LSD ($P=0.05$). Each value represents the mean of three replications per treatment. Error bars indicate $\pm$ standard error (SE). Here, C represents chitosan.

that rhizobacteria+chitosan biopreparation is more effective in controlling ToLCV as compared to rhizobacteria alone. Although the histopathological and histochemical studies revealed more destruction in the diseased plants, these changes could be reduced to the level of the healthy plants due to combined treatment of chitosan and *Pseudomonas* sp.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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