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Biocontrol of tomato leaf curl virus (ToLCV) in tomato with chitosan supplemented formulations of *Pseudomonas* sp. under field conditions

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Abstract

Of all the disease reported in tomato, the tomato leaf curl virus (ToLCV) disease is cited to be the most devastating both in terms of quantitative and qualitative yield losses. Often, the loss reaches to the extent of 100% during summer throughout India. Several majors have been taken to control the vector Whitefly. However, it has not been very effective in preventing epidemics and yield losses due to these viruses. There has been several researches done in this regard but so far, no economically viable management practices are available to manage ToLCV in tomato crop. Hence, the present investigation was carried out to evaluate previously proven potent rhizobacterial isolates against ToLCV. The investigations were carried out with potent rhizobacterial isolates alone and, along with elicitor molecules such as chitosan to determine its ability to control ToLCV in tomato. Different formulations of PGPR and chitosan were evaluated for controlling ToLCV and growth promotion of tomato under field conditions. A field experiment was laid out using a randomized block design (RBD) conducted in July, 2011, to study the efficacy of potential rhizobacterial strains along with chitosan in plant growth promotion and against ToLCV at a field in Dharwad, Karnatka, India. It was found that the application of *Pseudomonas* sp. in combination with chitosan reduces the severity of the ToLCV disease by 80.33–90.33 per cent. Application of chitosan or the bacterial inoculant alone was not effective to control the severity of ToLCV disease. However, the plants inoculated with the chitosan based formulation of *Pseudomonas* sp.(206(4) +B-15+ JK-16) recorded the highest activity of ISR molecules and recorded maximum plant height, total biomass, chlorophyll content, fruit number and yield over the diseased control. The quantification of viral load was done by semi quantitative PCR analysis which revealed the lowest viral load in plants inoculated with both chitosan and *Pseudomonas* sp. 206(4) + B-15+ JK-16. After application of chitosan, substantially higher number of bacterial cells on the roots was observed through scanning electron microscopy without any morphological and other qualitative differences. The results, thus, indicate that addition of chitosan has enhanced the biocontrol efficacy of *Pseudomonas* sp.against ToLCV.

Keywords: *Pseudomonas* spp; ToLCV; tomato; induced systemic resistance; chitosan,biocontrol; *Bemisia tabaci*; scanning electron microscopy.
Abbreviations: DAS_Days after sowing; DAI_Days after inoculation; ISR_Induced systemic resistance; PGPR_Plant growth promoting rhizobacteria; ToLCV_Tomato leaf curl virus.

Introduction

Tomato (*Solanum lycopersicon* L.) is an important and most widely grown vegetable crop in both tropics and sub tropics of the world. It ranks second in the importance among vegetables. Recently, there has been more emphasis on tomato production as it is not only considered as a source of vitamins, but also as a source of income and a major contributor towards food security. However, there are many constraints that come in the way of tomato production. Often, 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. In all reported viral diseases in tomato, tomato leaf curl virus (ToLCV), a geminivirus (Geminiviridae: subgroup – III), is the most important and destructive viral pathogen in many parts of India (Saikia and Muniyappa, 1989; Harrison et al., 1991). The incidence of ToLCV in tomato is growing in the areas of Karnataka ranging from 17 to 100 per cent in different seasons. And, 50 to 70 per cent yield loss is observed in tomato Cv. Pusa Ruby grown in February – May (Saikia and Muniyappa, 1989). The disease primarily caused by the transmission of insect vector "whitefly *Bemisia tabaci"* (Gennadius) (Homoptera: Aleyrodidae) (Muniyappa and Veeresh, 1984). The symptoms of disease become visible in tomato 2-3 weeks after infection and consist of upward curling of leaflet margins, yellowing of young leaves and abortion of flowers. Those leaflets that appear soon after inoculation are cupped down and inwards.Infected plants are severely stunted and resulting decrease of plant growth reduces total yield (Gafni, 2003; Crescenzi et al., 2004).

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 is known for growth promotion and disease reduction in crops.

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 and 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, pathogen-related (PR) proteins 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 to be active against viruses, bacteria and other pests (Abdelbasset et al., 2010). Based on these 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 potentials to control a variety of crops diseases. A number of plant growth promoting rhizobacteria have been implicated in the biocontrol of virus 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 by activating defense genes encoding Chitinase, beta-1, 3 glucanase, peroxidase, PALase and other enzymes (Srinivasan et al., 2005**;** Kirankumar, 2007) and chemicals (M'piga et al., 1997). However, effects of single biocontrol agents were often insufficient under practical conditions or difficult to reproduce (Punja and Yip, 2003). Combinations of different antagonists can overcome this problem, resulting in improved control of a pathogen or pathogen- complex (Raupach and Kloepper, 1998; Kim et al., 2008). The combined application of two or more biocontrol strains is likely to more closely mimic the natural situation and may, therefore, represent a more viable control strategy for tomato leaf curl virus. 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.

The aim of the present study is to compare the biocontrol ability of the selected rhizobacterial isolates against ToLCV disease, with or without PGPR amendments along with chitosan treatments; and to study the elicitation of ISR molecules the defense responses of plants induced by them.

Results

Ability of the rhizobacteria and chitosan to induce systemic resistance against ToLCV in tomato plants

Based on the initial screening, three isolates B-15, 206 (4) and JK-16 were selected for further characterization and biocontrol studies. Application of rhizobacteria along with chitosan significantly reduced ToLCV severity. 75 days after inoculation (75 DAI) of the pathogen, the maximum disease severity reduction of 90.33 per cent was observed in tomato plants treated with *Pseudomonas* sp. 206(4) + B-15+ JK-16+ Chitosan at 75 DAI (Fig 1). In the present study, higher level of accumulation of phenolics was observed in plants inoculated with *Pseudomonas* sp. 206(4) + B-15+ JK-16+ Chitosan compared to uninoculated plants. This strain recorded 41.66 per cent higher phenol content than the disease control (Fig 2). Inoculation of tomato plants with *Pseudomonas* sp. 206(4) + B-15+ JK-16+ Chitosan also resulted in the highest synthesis of phenylalanine ammonia lyase (32.87 per cent higher than the diseased control) (Fig 3). Though all the treatments tested in this investigation induced biosynthesis of peroxidase, *Pseudomonas* sp. 206(4) + B-15+ JK-16+ Chitosan showed the highest induction of peroxidase which was45.36 percent higher than the diseased control (Fig 4). Another fascinating observation was the enhanced chitinase activity in rhizobacteria treated plants (33.87 per cent higher than the diseased control) (Fig 5). Inoculation of tomato plants with *Pseudomonas* sp. 206(4) + B-15+ JK-16+ Chitosan also resulted in the highest synthesis of polyphenol oxidase activity (33.73 per cent higher than the diseased control) (Fig 6).

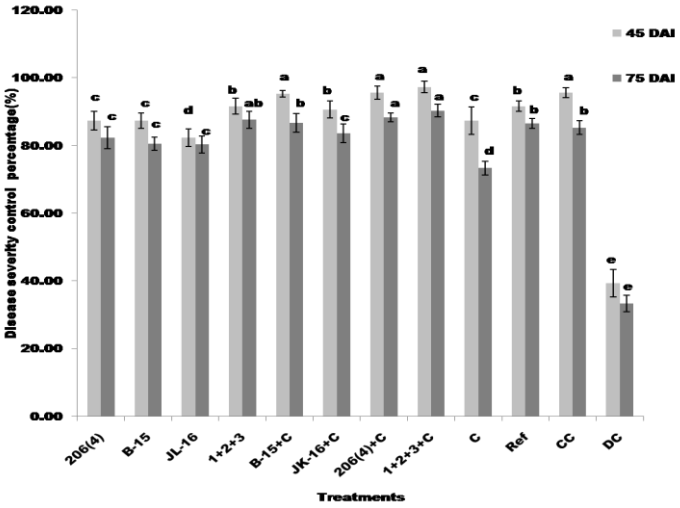

Fig 1. Biocontrol of ToLCV disease by the various selected rhizobacteria in combination with or without chitosan. Disease severity control % was observed at 45 and 75 days after innoculation (DAI) of viral pathogen. Different letters on bars indicate statistically significant between treated and control according to LSD ($P \leq 0.05$). Here, 1+2+3 =B-15+JK-16+206 (4), C=Chitosan, Ref=Reference strain, CC=Chemical control and DC = Disease control.

Detection of ToLCV inoculum in rhizobacteria and chitosan treated leaves

In order to quantify the viral inoculum in tomato plants treated with rhizobacterial isolates in combination with chitosan, a semi quantitative PCR analysis was carried out. This analysis detects and estimates the viral DNA accumulation in the challenge inoculated plants. All the rhizobacterial and chitosan treated plants and the disease control plants were analyzed by means of semi quantitative PCR. PCR reaction using coat protein gene specific primers were performed for different number of cycles *viz.*, 10, 20 and 30. PCR products were detected on agarose gel electrophoresis, and the threshold cycle was determined for

Table 1. Plant growth parameters of tomato as influenced by inoculation with rhizobacteria (at harvest) during Monsoon 2011.

Treatments	Plant height (cm)	Total biomass (Kg/plant)	Chlorophyll (SPAD)	No of Fruit/plant	Fruit yield (kg/plant)
Pseudomonas 206(4)	43.7 ±1.64 cd	0.037 ±0.061 d	32.7 ± 0.41 abcd	21.4 ±0.50 c	1.34±0.03 cd
Pseudomonas B-15	41.6 ±1.07 de	0.036 ±0.055 de	31.9 ±0.66 bcd	20.8 ±0.86 cd	1.25 ±0.08 d
Pseudomonas JK-16	40.2 ±0.33 de	0.035 ±0.041 e	30.8 ±0.84 d	18.8±0.86 de	1.07 ±0.03 e
Pseudomonas (206(4) +B-15+JK-16)	46.0 ±1.14 bc	0.040 ±0.067 b	34.7 ±0.72 ab	23.7±0.37 b	1.51 ±0.10 bc
Pseudomonas 206(4)+ Chitosan	48.2 ±1.64 b	0.042 ±0.051 a	34.2 ±0.62 ab	24.6±0.50 ab	1.60 ±0.08 b
Pseudomonas B-15 + Chitosan	43.6 ±1.37 cd	0.039 ±0.027 bc	33.8 ±0.78 ab	24.4 ±1.02 b	1.46 ±0.06 bc
Pseudomonas JK-16 + Chitosan	42.7 ±1.20 cde	0.038 ±0.044 c	33.1 ±0.89 abc	22.8±0.96 bc	1.32 ±0.08 cd
Pseudomonas (206(4) +B-15+JK-16) + Chitosan	52.5 ±1.38 a	0.043 ±0.041 a	35.2 ±1.02 a	26.6 ±0.81 a	1.77±0.0.07 a
Chitosan	39.7 ±1.39 e	0.033 ±0.044 f	30.5 ±0.56 cd	15.6±0.50 f	0.94 ±0.0.06 e
Reference strain (*P. fluorscencs* NCIM 2099)	43.4 ±0.95 cd	0.036 ±0.027 de	33.2 ±0.75 abc	23.0±0.70 bc	1.32 ±0.04 cd
Chemical control (confidor 2 ml/L)	35.7 ±0.94 f	0.031 ±0.035 g	30.7 ± 0.59 cd	17 ±0.70 ef	0.97 ±0.06 e
Diseased control (only ToLCV)	28.7 ±1.01 g	0.025 ±0.031 h	21.3 ±1.77 e	11.6± 0.51 g	0.45 ± 0.01 f

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 $P \leq 0.05$.

each treatment. In general, a slight reduction in the viral inoculums was observed in all most all the treated plants except disease control. All the plants treated with rhizobacterial isolates in combination with chitosan showed the viral inoculum lower than the plants received rhizobacterial treatment alone.The diseased control plant whicn showed the highest amount of viral load after 20th cycle of the PCR at 45 DAS.

At 75 DAS, all the treatments exhibited a significant amount of viral load after 10th cycle of the PCR, except rhizobacterial mixture + chitosan, *Pseudomonas* sp. 206(4) and chemical control treatment which showed the lowest amont of viral titre after 20th cycle of the PCR.The diseased control plant exhibited highest amount of viral load after 10th cycle of the PCR. At both 45 DAS and 75 DAS, *Pseudomonas* sp. 206(4) + B-15+ JK-16+ Chitosan showed the least viral load (Fig. 7).

Root colonization by Pseudomonas sp. 206(4) in presence and absence of chitosan

For achieving success in plant growth promotion and crop protection, there must be effective root colonization by the introduced microorganisms. SEM pictures reveal that tomato roots inoculated with *Pseudomonas* sp. 206(4) in combination with chitosan shows locally very dense population of bacteria. Here, the cells were observed encapsulated within a massive network of root derived material. Roots inoculated with *Pseudomonas* sp. 206(4) without chitosan was found to be exhibiting lesser population of bacteria (Fig. 8).However, no qualitative differences could be detected in the presence or absence of chitosan; i.e. cells exhibited similar size, same location and position towards the root surface. The synergistic effect of chitosan when applied together with the bacterial inoculant is intriguing. Here, the chitosan has stimulated the population of *Pseudomonas* sp.

Plant growth promotional potential of the selected rhizobacterial strains in combination with chitosan

The effect of rhizobacterial combination with chitosan on growth and yield parameters of tomato was studied. In

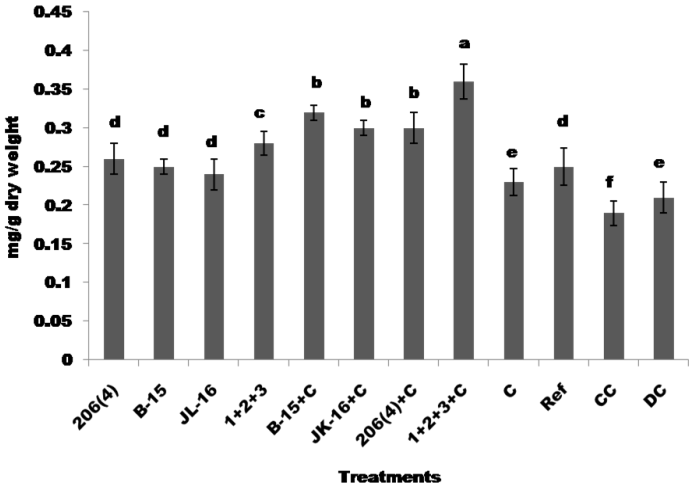

Fig 2. Effect of treatment with various selected rhizobacteria in combination with or without chitosan on phenol content in tomato leaves. Phenol content was estimated 45 days after sowing (DAS). Different letters on bars indicate statistically significant between treated and control according to LSD ($P \leq 0.05$). Here, 1+2+3 =B-15+JK-16+206 (4), C=Chitosan, Ref=Reference strain, CC=Chemical control and, DC=Disease control.

addition to suppressing the viral disease, *Pseudomonas* sp. 206(4) + B-15+ JK-16+ Chitosan greatly improved plant growth, biomass, chlorophyll content and yield (Table1). Inoculation of *Pseudomonas* sp. 206(4) + B-15+ JK-16+ Chitosan has significantly improved growth components of tomato. It has increased the plant height by 45.33 percent, biomass by 41.21 percent , chlorophyll content by over 39.48 and yield 74.57 percent compared to the virus inoculated control.

Discussion

We demonstrated the management of viral disease caused by ToLCV to agricultural system with the supplementation of biologically viable elicitiors, chitosan to enhance the activity

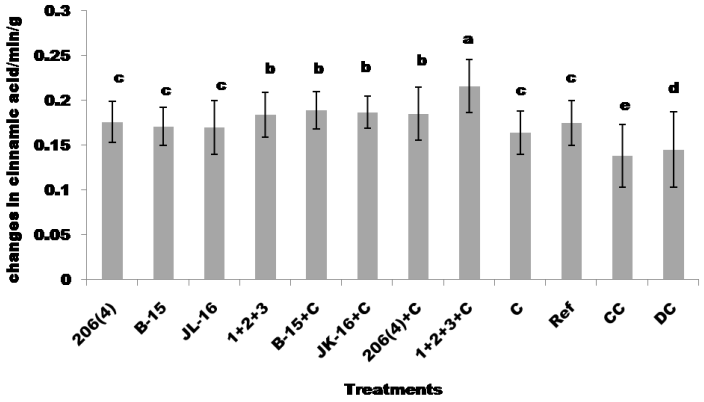

Fig 3. Effect of treatment with various selected rhizobacteria in combination with or without chitosan on phenylalaine ammonialyase activity in tomato leaves. Phenyl ammonia lyase activity was estimated 45 days after sowing (DAS). Different letters on bars indicate statistically significant between treated and control according to LSD ($P \leq 0.05$). Here, 1+2+3=B-15+JK-16+206 (4), C=Chitosan, Ref=Reference strain, CC=Chemical control and, DC=Disease control.

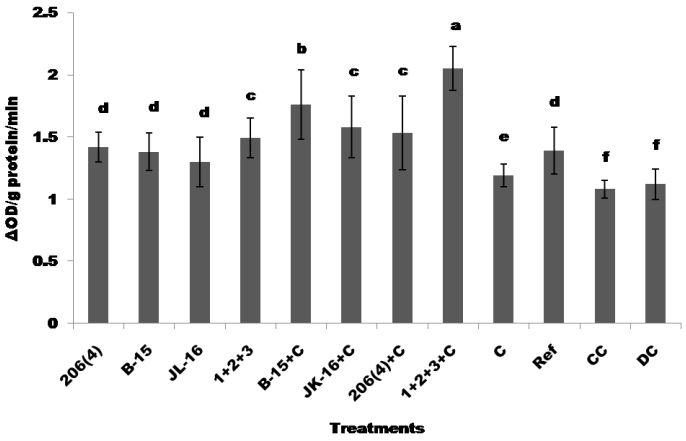

Fig 4. Effect of treatment with various selected rhizobacteria in combination with or without chitosan on peroxidase activity in tomato leaves. Peroxidase activity was estimated 45 days after sowing (DAS). Different letters on bars indicate statistically significant between treated and control according to LSD ($P \leq 0.05$). Here, 1+2+3 =B-15+JK-16+206(4), C=Chitosan, Ref=Reference strain, CC=Chemical control, DC=Disease and DC = Disease control.

of beneficial organisms, thereby increasing the resistance against pathogens. Thus, pest management practices are to be replaced by naturally occurring and introduced biocontrol agents. Towards this end, the biocontrol ability of rhizobacterial isolates was evaluated under high virus - vector pressure conditions. We initially demonstrated the biocontrol efficacy of chitosan treatment in the experimental strains of *Psedomonas Sp*., 206(4), B-15 and JK-16. The treatment management time of 75 days was selected in order to notice the observed changes after the treatment period with or without chitosan in the viral inoculated plants. Our results indicated that there was a significant reduction in the severity of the infected disease to the plants treated with chitosan compared to that of the untreated disease control.

Similarly, Postma et al. (2009) observed control of *Pythium aphanidermatum* in cucumber with a combined application of *Lysobacter enzymogenes* strain 3.1T8 and chitosan with reduction in number of diseased plants by 50-100 per cent. Murphy et al. (2000) also observed the biocontrol efficacy of *P. fluorescens* against CMV and tomato mottle virus in tomato under field conditions. Kandan et al. (2003) showed the biocontrol efficacy of *P. fluorescens* strains against tomato spotted wilt virus (TSWV) in tomato under field conditions.

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). Induction of defense response through the application of rhizobacteria often induces changes in defense molecules activity that are correlated with an increase in disease resistance, Higher level of phenolics was observed in plants inoculated with *Pseudomonas* sp. 206(4) + B-15+ JK-16 and chitosan compared to uninoculated plants. Our findings are in agreement with Kandan et al. (2003), who observed increase in phenolics in cowpea due to *P. fluorescens* inoculation which, in turn, protected plants from spotted wilt virus. In tomato, rhizobacterial treatment induced the plants to synthesize PAL, PO, Chitinase and PPO. 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). Cinammic 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 infectionof pathogen (Podile and Laxmi, 1998)**.** The increase in chitinase activity might have prevented the damage caused by viral pathogen and, thus, increased the per cent disease control in all the rhizobacteria treated plants. Synthesis and accumulation of PR proteins have been reported to play an important role 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; Van Loon, 1997). All the treatment induced biosynthesis of peroxidase, which are in agreement with the previous findings of Harish (2005), who observed control of banana bunchy top virus (BBTV) due to induction of increased peroxidase activity. Inoculation of tomato plants with *Pseudomonas* sp. 206(4) + B-15+ JK-16+ Chitosan also resulted in the highest synthesis of polyphenol oxidase activity .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). The PO and PPO activities are linked to lignifications 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).Similar kind of induced PPO activity was noticed by Kandan et al. (2005) when *P.fluorescens* strain CHAO applied alone or in combination with other strains to tomato seeds for controlling tomato spotted wilt virus in tomato. Thus, the observed induction of enzymes and the corresponding reduction in ToLCV infection in tomato supports the hypothesis that the resistance induced by isolates is systemic.

Chirkov et al (2001) reported callose and ribonuclease and β-1,3 glucanase induction in potato plants as defense respo-

nse against potato virus X (PVX) when plants were sprayed with chitosan solution (1 mg/ml). Nandeeshkumar et al. (2008) observed the enhanced activation of catalase, PALase, 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 fruit. Similar observations were reported with the potato virus X, tobacco mosaic and necrosis viruses, alfalfa mosaic virus, peanut stunt virus, and cucumber mosaic virus (Pospieszny et al., 1991; Chirkov et al., 2002). On bean leaves, local infections produced by alfalfa mosaic virus (ALMV) 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 tomato leaves treated with chitosan at the same concentration and inoculated with potato spindle tuber viroid (Pospiezny,1997). In these studies, systemic resistance was induced by chitosan on various host virus combinations. In general, it was observed that chitosan treatments along with PGPR significantly reduced virus infection in various plants.

The semi quantitative PCR was used to detect and estimate the viral DNA load in plants. The leaves of rhizobacteria-treated and untreated tomato plants were tested for the presence of the virus. In general, a slight reduction in the viral inoculum load was observed in all the rhizobacteria and chitosan treated plants. 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.

The results of present investigation showed that chitosan supplementation resulted in an increase in the antagonists population on tomato roots. The fact that chitosan is a rich N-source as well as a C-source may be significant here. The increased numbers of antagonists on the roots are probably explained by the role of chitosan as a nutrient source. These findings are completely supported by Postma et al. (2009) who observed substantially higher number of bacterial cells (*Lysobacter enzymogenes* 3.1T8) in cucumber roots by scanning electron microscopy which enhanced the biocontrol efficacy of *L. enzymogenes* 3.1T8 against crown rot in cucumber.

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). Manjula and Podile (2005) reported, 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.

Materials and Methods

Raising Tomato seedlings

Tomato seeds (var., Pusa Ruby) were sown in plastic pots filled with soil and farmyard manure. Twenty five days old seedlings were used for viral inoculation purposes.

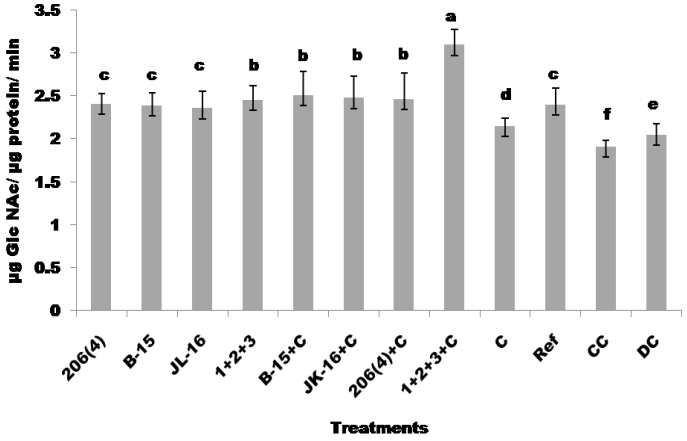

Fig 5. Effect of treatment with various selected rhizobacteria in combination with or without chitosan on chitinase activity in tomato leaves. Chitinase activity was estimated 45 days after sowing (DAS). Different letters on bars indicate statistically significant between treated and control according to LSD ($P \leq 0.05$). Here, 1+2+3 =B-15+JK-16+206(4), C=Chitosan, Ref=Reference strain, CC=Chemical control and, DC=Disease control.

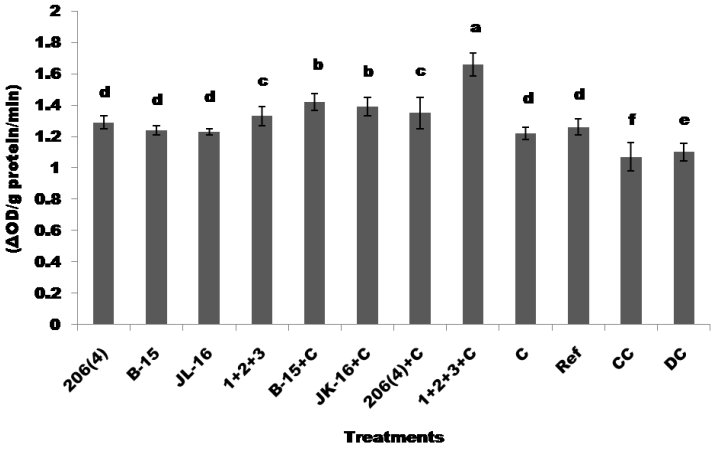

Fig 6. Effect of treatment with various selected rhizobacteria in combination with or without chitosan on polyphenol oxidase activity in tomato leaves. Polyphenol oxidase activity was estimated 45 days after sowing (DAS). Different letters on bars indicate statistically significant between treated and control according to LSD ($P \leq 0.05$). Here, 1+2+3 =B-15+JK-16+206(4), C=Chitosan, Ref=Reference strain, CC=Chemical control and, DC=Disease control.

Maintenance of the viral inoculum

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 were maintained in the glass house throughout the period of study.

Vector culture rearing

Whiteflies were collected from cotton and tobacco plants from fields by sucking with the help of an aspirator and released on to the ToLCV diseased tomato plants grown in insect proof rearing cages and continuously maintained.

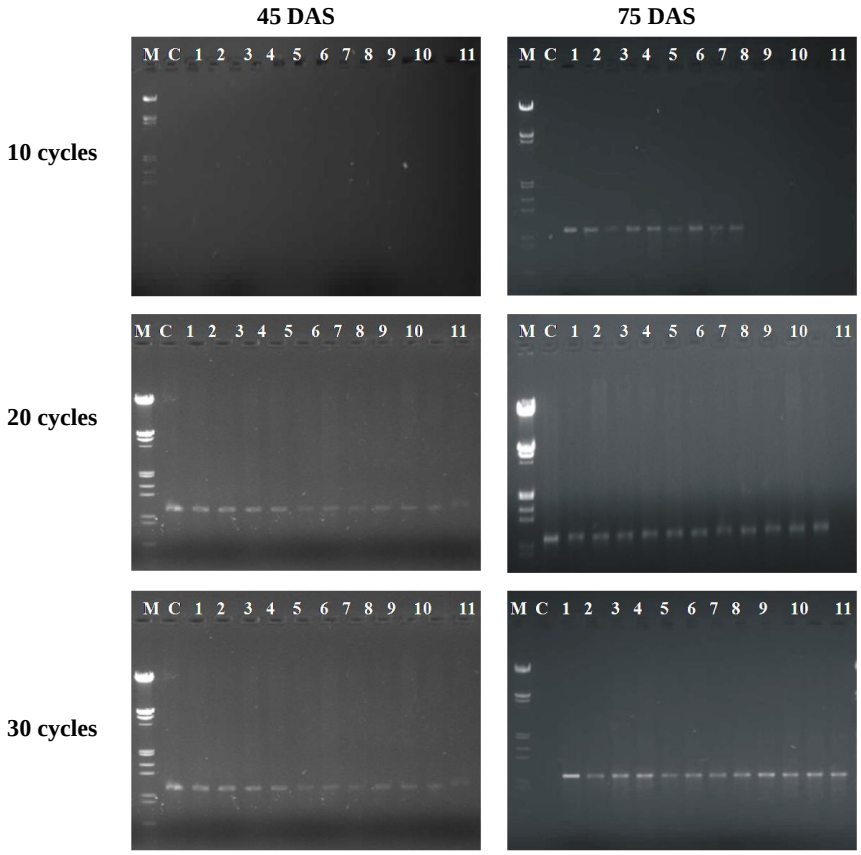

Fig 7. Semi-quantitative PCR analysis of ToLCV in tomato. Here, Lane M : Double digest marker (*ECoR*I+*Hind* III), Lane C: ToLCV amplicon after 10, 20 and 30 cycles (control), Lane 1-11: PGPR treated plants with and without chitosan after 10, 20 and 30 cycles; (C) Disease control, (1) B-15, (2) 206 (4), (3) JK-16, (4) Chitosan, (5) B-15+206(4)+ JK-16, (6) Reference strain, (7) B-15+Chitosan, (8) JK-16 + Chitosan, (9) 206 + Chitosan, (10) B-15+206(4)+ JK- 16+Chitosan, (11) 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.

Release of viruliferous insects

The viruliferous insects were sucked from the diseased plants using an aspirator and released on to healthy and 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. Twenty five days old seedlings were used for release of the viruliferous insects. Thus, it was ensured that all the seedlings were infected with ToLCV.

Rhizobacterial isolates

Three efficient plant growth promoting rhizobacteria (PGPR) (*Pseudomonas* 206(4), *Pseudomonas* B-15 and *Pseudomonas* JK-16), selected after preliminary screening against ToLCV (Shefali, 2012) were used in the studies.

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, UAS, Dharwad, during Kharif season (June-Dec. 2011). Five weeks old seedlings of variety Pusa Ruby, raised in a glasshouse were transplanted in the main field with plot size 25m x 12 m, following a spacing of 75cm x 60 cm. 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. 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.

Rhizobacterial treatment

The bacteria were grown in King's B broth medium on a shaker (150 rpm) for two days, centrifuged at 10,000 rpm for 5 min and the pellet mixed with sterile carboxy methyl cellulose suspension (1%). Tomato seeds were surface sterilized with sodium hypochlorite solution, placed in CMC cell suspension, air dried inside a laminar flow chamber and the biocoated seeds sown in pots. For soil application, the lignite based culture 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. Control plants in pots without application of rhizobac-

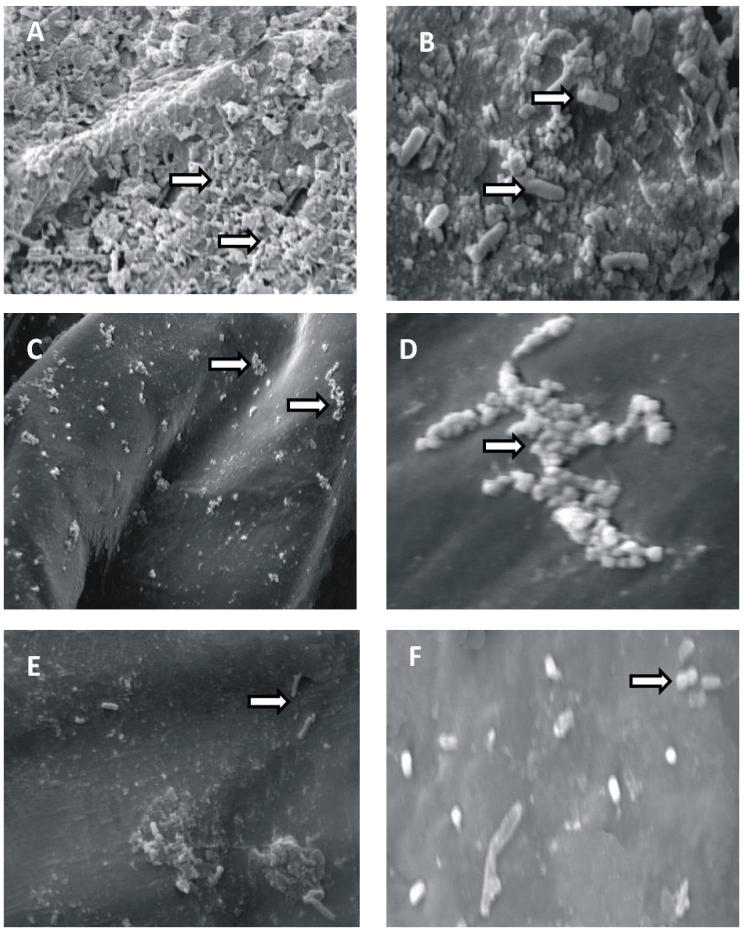

Fig 8. Scanning Electron Microscopy photographs of *Pseudomonas* sp. on tomato roots in combination with (A-D) or without chitosan (E-F). Roots inoculated with *Pseudomonas* sp. 206(4) in combination with chitosan showed locally very dense population of bacterial cells on root surface (A-B). The cells were encapsulated within a massive network, probably being root derived material (C-D).Roots inoculated with *Pseudomonas* sp. 206(4) but without chitosan also contained bacterial cells, but much less(E-F). Arrows point at bacterial cells on root surface.

teria were also maintained. All treatments were replicated thrice and arranged in a randomized complete block design (RCBD).

Chitosan treatment

Chitosan was dissolved in 100 mM acetate buffer (pH 4.5) and the pH adjusted to 6.5 using 1N NaOH. The bacterial cell pellet was mixed well with chitosan solution (5%). Surface sterilized Tomato seeds were soaked in chitosan cell suspension and shaken for 3 h at 28^{o}C. 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 adjusted with 1 N NaOH to pH 6.5.

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 and ground to a powder. The powdered sample was extracted with 10 ml of chilled 0.1M phosphate buffer (pH 7.0) in a pestle and mortar, centrifuged at 18,000 rpm at 5°C for 30 min. and the supernatant used as the enzyme source for the estimation of peroxidase enzyme (Mahadevan and Sridhar, 1986), PALase (Ross and Sederoff, 1992), Poly phenol oxidase (Mayer et al., 1965), chitinase activity (Miller, 1959).The total phenol content in leaves was estimated following Folin Cio-calteau method (Sadasivam and Manickam, 1991).

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).

Semi-quantitative PCR 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.

Visualization of Pseudomonas sp. on tomato roots by SEM

To determine the location of *Pseudomanas* 206(4) on the roots and to study if any morphological differences occurred due to application of chitosan, Scanning Electron Microscopy (SEM) studies were conducted. This was carried out at RUSKA Lab, College of Veterinary Science, SVVU, Rajendranagar, Hyderabad, India.

The surface sterilized Tomato seeds were allowed to germinate in plastic cups filled with sterlized soil and sand and the cups incubated in a glass house at optimum conditions. Ten days after sowing, the plants were inoculated with *Pseudomanas* 206(4) with and without chitosan (5%) and incubated in a glasshouse at optimum conditions. The roots were sampled five days later and fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) at 4°C for 24 h and post fixed in 2% aqueous osmium tetraoxide for 1 h. They were dehydrated in series of graded alcohols and dried to critical point drying with CPD unit. The processed samples were mounted over the silver stubs (metallic holder) fixed with double sided carbon conductivity tape and subjected for gold sputtering by using an automated sputter coater (JEOL JFC-1600) for 3 minutes at 40 psi and the specimens observed under a Scanning Electron Microscope ("JEOL"-JSM 5600, Japan) at various magnifications and the photographs taken.

Statistical analysis

All treatments were replicated thrice and arranged in a randomized complete block design (RCBD).All data in the present study were subjected to the analysis of variance (ANOVA) and means were separated by Duncan's multiple range tests. Means were compared using the least significant difference (LSD) $p \leq 0.05$.

Conclusion

The study has brought out the biocontrol potential of the selected combination of PGPR with chitin supplemented formulations enhanced the efficacy of PGPR in reducing the disease severity of ToLCV as well as plant growth promotion and yield in tomato essentially through either 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, and the elicitation of biochemical defense responses. Thus, the present research showed that the mixture of rhizobacterial isolates combined with chitosan is a potential biocontrol treatment with reproducible biocontrol effects on ToLCV in tomato.

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