

Full Length Research Paper

Plant growth promoting rhizobacteria (PGPR) induces resistance against *Fusarium* wilt and improves lycopene content and texture in tomato

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Plant growth promoting *Bacillus subtilis* (BS2) was found effective against tomato wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* under field conditions. Pretreatment of tomato plants with *B. subtilis* BS2 significantly induced the activities of defense related enzymes viz., peroxidase, polyphenol oxidase, chitinase and phenylalanine ammonialyase and phenolics when challenged with the pathogen. Apart from disease control, BS2 improved the fruit quality with high lycopene (76.30 mg/kg against control, 40.34 mg/kg) and texture (90.5 F_{max} against control, 56.35 F_{max}) during harvest and even 15 days after harvest, similar trend was maintained unequivocally indicating that plant growth promoting rhizobacteria (PGPR) can improve the nutritional quality as well as shelf life of the fruits.

Key words: Plant growth promotion, plant growth promoting rhizobacteria (PGPR), defense enzymes, lycopene, fruit texture.

INTRODUCTION

Tomato is an important worldwide cultivated solanaceous vegetable playing a major role in human nutrition. Wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* is a serious concern in tomato cultivation (Loganathan et al., 2009). Management of the disease through an eco-friendly approach will nullify the ill effects such as environmental pollution, residual toxicity and fungicidal resistance in pathogens due to use of chemicals. Many bacterial communities living in the rhizosphere and found associated with plant growth promotion activity are called plant growth promoting rhizobacteria (PGPR) (Kloepper

et al., 1980). The PGPR are known to act as biocontrol agents through competition for ecological niche/substrate and production of siderophores, antibiotics, hydrogen cyanide and fungal cell wall lysing enzymes (Glick and Bashan 1997; Wang et al., 2000; Saravanakumar et al., 2007). PGPR are also involved in induced systemic resistance (ISR) against many diseases in a wide range of crops (Kloepper and Beauchamp, 1992; Liu et al., 1995; Chen et al., 2000; Sangeetha et al., 2010). Although the application of PGPR and their effects on disease or pest control and yield have been well

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established for a wide range of crops, information on fruit quality in terms of lycopene content and texture is very limited. Lycopene, a carotenoid present in tomato is an important compound in human nutrition as it plays a key role in reducing cardiovascular as well as prostate cancer diseases (Giovannucci, 1999; Giovannucci et al., 2002). Apart from the medicinal value, the market quality of the tomato fruit is also affected by lycopene because fruit color is determined by carotenoids, and primarily lycopene (Fraser et al., 1994; Shi and Le Maguer, 2000). Shelf life of tomato fruits is dependent on fruit firmness. Main factor affecting firmness of the fruit during maturity is microbial spoilage (Kramer et al., 1992). In our earlier study we identified two potential PGPR: *Bacillus amyloliquefaciens* isolate BA1 (earlier reported as chilli 1) and *Bacillus subtilis* isolate BS2 (earlier reported as chilli 2), based on preliminary studies related to plant growth promotion and plant disease control (Loganathan et al., 2010). In that study, BA1 and BS2 manifested high level of *in vitro* efficacy in producing plant growth promoting compounds (indole acetic acid and siderophores) and solubilizing phosphates apart from inhibiting mycelial growth of wilt fungus, *F. oxysporum* fsp. *lycopersici*. In the present study, field efficacy of PGPR bio-formulations (BA1 and BS2) on *Fusarium* wilt and quality of fruits (lycopene content and texture) during harvest and post-harvest were determined.

MATERIALS AND METHODS

Testing the talc bio-formulation efficacy under field conditions

Talc formulation of PGPR isolates BA1 and BS2 were prepared using a standard protocol (Vidhyasekaran and Muthamilan, 1995) with some minor modifications. The PGPR isolates were cultivated in nutrient broth (HiMedia Laboratories Pvt. Ltd., Mumbai, India) for 48 h at temperature of $28\pm 2^\circ\text{C}$ under shaking at 20 g. Four hundred milliliters of bacterial culture containing 8×10^8 cfu/ml was mixed with 1 kg talc (Talc India, Rajasthan, India) sterilized at 105°C for 12 h along with 10 g carboxy methyl cellulose (adhesive agent) and 15 g calcium carbonate (to neutralize the pH to 7.0). The bio-formulation was shade dried for 12 h at ambient temperature ($28\pm 2^\circ\text{C}$) and packed in polythene packs. Bacterial population in the talc bio-formulation at the time of application was 6×10^8 cfu/g. Two field trials were conducted in years 2009 (trial I at Upland Block at Indian Institute of Vegetable Research, Varanasi, India) and 2010 (trial II at Lowland Block at Indian Institute of Vegetable Research, Varanasi, India). Both blocks have recorded 40-60% *Fusarium* wilt incidence in tomato (cv DVRT-1) in the previous year. The seeds of tomato (cv. DVRT-1 obtained from Seed Production Unit, Indian Institute of Vegetable Research, Varanasi, India) were treated with the talc formulation (10 g formulation/kg of seeds) and shade dried for 12 h. The treated seeds were sown in nursery beds applied with talc formulation (mixed thoroughly 50 g talc formulation with 5 kg well decomposed Farm Yard Manure (FYM) and applied to the 3m^2 bed). In chemical treatment, seeds treated with 1 g of carbendazim 50% WP (Dhanuka Agritech Ltd., Gurgaon, India) per kg of seeds were sown in nursery beds applied with well decomposed FYM (5 kg/ 3m^2). In control, nursery beds were prepared as described in chemical treatment but seeds were sown without any treatment. After 25 days, under bio-formulation treatment, main field was applied with 2.5 kg bioformulation in 50 kg

of well decomposed FYM ha^{-1} . The seedlings under bio-formulation treatment were uprooted and the root portion was dipped in talc based bio-formulation solution (1.0%) for 30 min and transplanted in the main field. Similarly, for the chemical and untreated controls, the seedlings were dipped for 30 min in 0.1% carbendazim solution and water respectively and transplanted separately in the main field and FYM alone (50 kg/ha) was applied. The experiment was conducted in randomized block design with four replications of each treatment. Observations on disease control and measurement of plant height were recorded 45 days after transplanting.

Testing quality of fruits

In all the treatments the mature fruits were harvested from the field separately and analyzed for fruit texture and lycopene content. In tomato, third harvest normally gives maximum yield since it matches with exact maturity. Hence third harvest fruits were used in all fruit quality analyses. For each of these analyses, five fruits were randomly collected from each replication (in each treatment there were four replications hence total number of fruits were 20/treatment).

Analysis of fruit texture

Texture of the fruit was analyzed by a puncture test using a Texture Analyzer TA-XT (Stable Micro Systems), loading (2 mm s^{-1}) at a distance of 15 mm on two opposite points along the equatorial plane (Mena-Violante and Olalde-Portugal, 2007). Finally an average maximum force (F_{max}) per fruit was calculated.

Estimation of lycopene

Lycopene content was estimated by a spectrophotometric method as described by Anthon and Barrett (2001). The samples were extracted with a solution containing ethanol and hexane (4:3 v/v) and the phases were separated. Amount of light absorption were recorded from hexane phase at 503 nm and the results were interpreted using the value of 172/nM as the extinction coefficient for lycopene in hexane and expressed in mg/kg of fruits

Sample collection for analyses of induced defense proteins and phenolics

Pot mixture (consists of red soil : sand : well decomposed FYM at 1:1:1 ratio) was autoclaved for 1 h for two consecutive days at 24 h interval and filled in pots (20 cm diameter and 30 cm height). Virulent isolate of *F. oxysporum* f.sp. *lycopersici* (FOL) was mass multiplied in sterilized sand-maize (19:1 ratio) medium for 15 days and mixed in the pot soil (10 g sand maize fungal culture/kg of soil). Tomato seeds were treated with talc formulation as described in field experiments and sown on sterilized soil. After 24 days, the seedlings were uprooted and the root portions of the seedlings were dipped in 1.0% talc formulation solution for 30 min. The treated seedlings were transplanted into the pot containing sterilized pot mixture treated with PGPR talc formulation (10 g/kg of soil) with or without pathogen according to the treatment details mentioned in Table 5. For analysis of defence related enzymes and chemical, root portion of 45 days old seedlings (21 days after transplanting) from different treatments was used. Five seedlings per replication were uprooted and thoroughly washed with running tap water. Root portions were removed from the seedlings and cut into pieces and powdered using liquid nitrogen and the frozen powder was used for each analysis. The protein content of sample was estimated by Lowry et al. (1951) using bovine serum albumin

as standard.

Assay of phenylalanine ammonia-lyase (PAL)

One gram of plant root sample was homogenized in 3 ml of ice cold 0.1 M sodium borate buffer (pH 7.0) containing 1.4 mM of 2-mercaptoethanol and 50 mg of insoluble polyvinylpyrrolidone (PVP) by using pestle and mortar. The extract was filtered through cheese cloth to remove debris, and centrifuged at 20,000 g for 10 min at 4°C and the supernatant was used as the enzyme source. PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm (Dickerson et al., 1984) and was expressed in nmol trans-cinnamic acid min⁻¹ mg⁻¹ of protein

Assay of chitinase

Plant root sample (1 g) was extracted with 5 ml of 0.1 M sodium citrate buffer (pH 5.0) and the content was centrifuged at 20,000 g at 4°C for 10 min and the supernatant was used as enzyme source. The colorimetric assay of chitinase was carried out as described by Boller and Mauch (1988) and the enzyme activity was expressed as nmoles GlcNAc equivalents min⁻¹ mg⁻¹ protein.

Assay of peroxidase (PO)

One gram of plant root sample was homogenized with 2 ml of 0.1 M phosphate buffer (pH 7.0) at 4°C and then centrifuged at 15,000 g and 4°C for 15 min. The supernatant (0.5 ml) was mixed with 0.5 ml of 0.05 M pyrogallol and 0.5 ml of 1% H₂O₂ and incubated at room temperature (28 ± 2°C). The absorbance was recorded at 420 nm at 30 s intervals up to 3 min and the activity was expressed as changes in the absorbance min⁻¹ mg protein⁻¹ (Hammerschmidt et al., 1982).

Assay of phenol

Root samples (1 g) were homogenized in 80% methanol as described by Zieslin and Ben-Zaken (1993). The reaction mixture consisting of 1 ml methanolic extract, 5 ml distilled water and 250 µl Folin-Ciocalteu reagent (1 N) was prepared and kept at 25°C. The development of blue colour was measured at 725 nm and compared with standard catechol and the amount of phenolics was expressed as µg catechol mg protein⁻¹.

Native PAGE analysis of polyphenol oxidase (PPO)

The effect of PGPR treatment on induction of PPO isoforms was visualized in native PAGE electrophoresis (Laemmli 1970). Root tissue (1 g) was homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) at 4°C and centrifuged at 20,000 g for 10 min at 4°C and the supernatant was used as enzyme extract. Sample (60 µg) containing protein was loaded in polyacrylamide gel (8%). After electrophoresis, the gel was equilibrated in 0.1% *p*-phenylene diamine in 0.1 M potassium phosphate buffer (pH 7.0) for 30 min followed by 10 mM catechol in the same buffer. Finally, the PPO isoforms were visualized by adding catechol which resulted in appearance of dark brown discrete bands (Jayaraman et al., 1987).

Statistical analysis

Data were statistically analyzed (Gomez and Gomez, 1984) and the

treatment means were compared by Duncan's multiple range test (DMRT). The package IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, Philippines was used in all the analyses.

RESULTS

Efficacy of PGPR isolates against *Fusarium wilt* disease of tomato under field conditions

In both trials (trials I and II), *B. amyloliquifaciens* BA1 and *B. subtilis* BS2, demonstrated substantial wilt reduction as compared to the untreated control, and served as good or better wilt control than the chemical treatment. Comparing the two isolates, BS2 showed significantly better field efficacy against FOL than BA1 in the trials (Table 1). In trial I, BS2 treatment has less incidence of wilt as compared to BA1, chemical and control treatments (16.0, 25.3, 26.0 and 45.2%, respectively; P<0.05). Similarly in trial II, BS2 showed significantly less wilt incidence (24.6%) and the effect was statistically at par with the chemical control (23.8%) and better than BA1 (28.0%). Pooled mean comparison of both trials revealed that BS2 registered 58.4% wilt reduction over control which was greater than the chemical control (48.97%). Observations on yield parameters revealed that BS2 recorded the highest yield (43.90 t/ha) followed by BA1 (38.50 t/ha) and chemical (32.5 t/ha) while the least quantity was recorded in control (26.85 t/ha) (Table 2). The yield parameter had a positive relationship with plant growth as it was highest in BS2 (57.7 cm) followed by BA1 (51.95 cm), chemical (48.15 cm) and control (44.80 cm).

PGPR treatment on quality of fruits

Fruits collected at different harvesting periods were analyzed for lycopene content and texture (Tables 3 and 4). Lycopene content was increasing invariably towards maturity of the plants irrespective of the treatments. However, under PGPR treatments the content was significantly higher than in the chemical and untreated control. Among the treatments, BS2 recorded the highest content in all the harvest (71.28 mg/kg) when compared with BA1 (46.21 mg/kg), chemical (39.12 mg/kg) and control (35.21 mg/kg). Apart from this, fruits were analyzed for texture and lycopene content at the time of harvest and after 15 days of harvest. Results indicate that in all cases, there was a decline in texture profile after 15 days of storage while lycopene content increased (Table 4). At harvest, the highest texture profile (F_{max}) was recorded in plants inoculated with BS2 (90.5) followed by BA1 (79.16), carbendazim (61.96) and control (56.35). After 15 days, there was a declining trend in F_{max} value in all the treatments but in BS2 the content was high (11.10) as compared to control (2.14) and other

Table 1. Effect of PGPR treatment on fusarial wilt disease of tomato under field conditions.

Treatment	**Wilt incidence (%)		Mean wilt incidence (%)	Wilt reduction over control (%)
	Trial I	Trial II		
BA-1	25.30 ^b	28.00 ^b	26.65	45.39
BS-2	16.00 ^a	24.60 ^a	20.30	58.40
Carbendazim	26.00 ^b	23.80 ^a	24.90	48.97
Control	45.20 ^c	52.40 ^c	48.80	0.00

Means in a column followed by the same superscript letters are not significantly different according to Duncan's multiple range test at P=0.05. **Data were transformed using arcsine and analyzed

Table 2. Effect of PGPR on plant growth and yield of tomato under field conditions.

Treatment	Plant height [^] (cm)		Mean	*Yield (t/ha.)		Mean
	Trial I	Trial II		Trial I	Trial II	
BA-1	56.3 ^b	47.6 ^b	51.95	45.00 ^b	32.00 ^b	38.50
BS-2	62.1 ^a	53.3 ^a	57.70	49.20 ^a	38.60 ^a	43.90
Carbendazim	50.0 ^c	46.3 ^b	48.15	36.00 ^c	29.00 ^c	32.50
Control	47.6 ^c	42.0 ^c	44.80	32.10 ^d	21.60 ^d	26.85

[^]Plant height measured during the 3rd harvest; *Cumulative yield of three harvests; Means in a column followed by same superscript letters are not significantly different according to Duncan's multiple range test at P=0.05.

Table 3. Effect of PGPR treatment on lycopene content at different stages of fruit harvest.

Treatment	Lycopene content (mg/kg)			Mean
	First harvest	Second harvest	Third harvest	
BA-1	45.00 ^b	46.00 ^b	47.63 ^a	46.21
BS-2	64.10 ^a	73.44 ^a	76.30 ^a	71.28
Carbendazim	37.00 ^c	37.36 ^c	43.00 ^b	39.12
Control	30.30 ^d	35.00 ^c	40.34 ^b	35.21

Means in a column followed by same superscript letters are not significantly different according to Duncan's multiple range test at P=0.05.

Table 4. Effect of preharvest application of PGPR on post harvest fruits quality.

Treatment	Texture profile analysis (TPA) force (F_{max})		Lycopene content (mg/kg)	
	At harvest*	15 days after harvest	At harvest*	15 days after harvest
BA-1	79.16 ^b	7.86 ^b	47.63 ^b	48.34 ^b
BS-2	90.50 ^a	11.10 ^a	76.30 ^a	82.70 ^a
Carbendazim	61.96 ^c	4.23 ^b	43.00 ^c	48.65 ^b
Control	56.35 ^d	2.14 ^c	40.34 ^d	44.70 ^c

*Fruits collected from 3rd harvest were used; Means in a column followed by same superscript letters are not significantly different according to Duncan's multiple range test at P=0.05.

treatments. Post harvest lycopene analysis indicated that there was an increase in the content after 15 days of storage in all the treatments (Table 4) but the increase

was greater in BS2 treatment (82.70 mg/kg) followed by carbendazim (48.65 mg/kg), BA1 (48.34 mg/kg) and control (44.70 mg/kg).

Table 5. Induction of defense related enzymes and phenol in tomato plants challenged with FOL.

Treatment	Phenylalanine ammonia lyase (nmol of trans-cinnamic acid min ⁻¹ mg ⁻¹ of protein)	Peroxidase (Changes in the absorbance min ⁻¹ mg ⁻¹ of protein)	Poly phenol oxidase (Changes in the absorbance min ⁻¹ mg ⁻¹ of protein)	Chitinase (nmol of GlcNAc mg ⁻¹ min ⁻¹)	Phenol (µg catechol mg of protein)
BA1	23.33 ^d	1.13 ^c	1.63 ^c	5.61 ^b	43.00 ^b
BS2	25.60 ^c	1.00 ^c	1.40 ^d	4.73 ^{bc}	43.00 ^b
BA1+FOL	29.10 ^b	1.90 ^a	1.84 ^b	9.71 ^a	58.64 ^a
BS2+FOL	30.53 ^a	1.96 ^a	2.60 ^a	11.30 ^a	59.30 ^a
FOL	22.00 ^d	1.63 ^b	1.50 ^d	6.33 ^b	45.63 ^b
Healthy plant	20.00 ^e	0.80 ^d	1.30 ^f	3.00 ^c	42.30 ^b

Values are the means of three replicates; Means in a column followed by same superscript letters are not significantly different according to Duncan's multiple range test at P=0.05.

Induction of defense related enzymes

Induction of phenyl alanine ammonia lyase (PAL), peroxidase (PO) polyphenol oxidase (PPO), chitinase and phenol was observed in plants treated with PGPR upon challenged with FOL (Table 5). Induction of PAL was more in PGPR treated plants challenged with FOL and the effect was greater in BS2 (30.53 nmol min⁻¹ mg⁻¹) followed by BA1 (29.10 nmol min⁻¹ mg⁻¹), pathogen inoculated control (22.00 nmol min⁻¹ mg⁻¹) and untreated control (20.00 nmol min⁻¹ mg⁻¹). Similarly, all PGPR treated plants challenged with pathogen showed high level induction of PO, chitinase and phenol as compared to the control. However, in the case of PPO, the induction was significantly high in BS2 treated plants challenged with pathogen followed by BA1 and other treatments.

Native PAGE analysis of tomato plants treated with BS2 with or without pathogen inoculation showed four isoforms (PPO1, PPO2, PPO3 and PPO4) in BS2 treated plants challenged with FOL (Figure 1). Among the four isoforms, PPO3 and PPO4 were induced commonly in healthy control, pathogen inoculated control and BS2 with or without pathogen treated plants. On the other hand, PPO2 was absent in healthy and present in the other treatments and PPO1 was exclusively present in BS2 treated plants challenged with pathogen. Presence of strong signal of PPO3 and PPO4 isoforms was observed in the BS2 treated plants challenged with pathogens.

DISCUSSION

Majority of PGPR isolated for the management of plant diseases are *Pseudomonas* spp., (Ramamoorthy et al., 2002) or *Bacillus* spp., (Schisler et al. 2004). In our preliminary study, out of 142 rhizospheric PGPR tested, two *Bacillus* spp.: *B. amyloliquefaciens* and *B. subtilis* were identified as potential isolates for use against fungal pathogens such as *F. oxysporum* f.sp. *lycopersici* and *Sclerotium rolsii* apart from plant growth promotion through production of indole acetic acid and phosphate

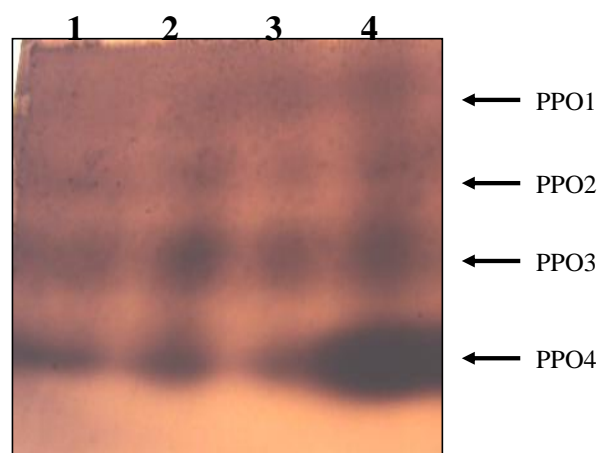


Figure 1. Induction of PPO isozyme in tomato plant treated with BS2 treatment with or without FOL, where lanes 1, Healthy; 2, FOL inoculated control; 3, BS2 treatment alone; 4, BS2 treatment challenged with FOL.

solubilization (Loganathan et al., 2010). In the present study, among the two isolates (BS2 and BA1), BS2 proved to be more effective against wilt pathogen, *F. oxysporum* f.sp. *lycopersici* under field conditions. Similarly, PGPR were reported to control fungal diseases caused by soil borne and leaf spot pathogens in wide range of crops (Wei et al., 1996; Viswanathan and Samiyappan, 2001; Ramamoorthy et al., 2002; Vivekananthan et al., 2004).

In the present study, the lycopene content in DVRT1 was found to increase from first harvest onwards and reached maximum at the third harvest. However, the influence of PGPR treatment, especially BS2 on lycopene content was greater since in all three harvests, the content was significantly higher than other treatments indicating that BS2 has a pivotal role in enhancing the content. It has been reported that environment conditions and plant nutrient status played a major role on status of lycopene content (Abushita et al., 2000; Binoy et al.,

2004). By keeping environmental conditions constant, there was a clear difference between PGPR treated and untreated plant fruits indicating the direct or indirect influence or both on lycopene content. Apart from lycopene, important quality of fruit is shelf life or post harvest keeping quality. Fruits from the BS2 treated plot exhibited considerably high level texture even at well ripening stage (15 days after harvest). Fruits with high texture showed extended keeping quality. The fruit texture is associated with low production ethylene production (Alexander and Grierson, 2002) and it has been demonstrated in plants treated with PGPR (Glick et al., 1998). In the present study, alternation of ethylene production is indirectly reflected in promoting growth of the tomato plants (Table 2). Though there is a relationship between PGPR treatment and improvement of fruit texture, further research in this line is required. This has been found to be of significant importance since less textured/firm fruits are more prone to spoilage by microbes (Mena-Violante and Olalde-Portugal, 2007).

Application of PGPR for plant disease management is gaining importance, due to induction of defense related enzymes (Viswanathan and Samiyappan, 2001) and chemicals (Chen et al., 2000) to suppress the pathogen thereby enabling a process called induced systemic resistance (ISR). In the present study, PAL, PO, PPO, chitinase and phenol were triggered in PGPR treated plant inoculated with FOL indicating activation of ISR mechanism by PGPR. The defense related enzymes play a major role in biosynthesis of lignin, oxidation of phenol and synthesis of antimicrobial phytoalexins (Daayf et al., 1997). The defense reaction was observed in several crops against a wide range of pathogens. Tomato plants treated with *Pseudomonas fluorescens* Pf1 recorded high level induction of defence enzymes in response to *Pythium aphanidermatum* attack (Ramamoorthy et al., 2002). Similarly, mango trees sprayed with *P. fluorescens* FP7 recorded a greater amount of the enzymes against *Colletotrichum gloeosporioides* infection (Vivekanadan et al., 2004). Mixture of *Pseudomonas* (NFP6), *P. fluorescens* (Pf3a), and *B. subtilis* (BS1) was found to induce defence reaction in banana against crown rot caused by *Lasiodyplodia theobromae* and *Colletotrichum musae* under *in vivo* conditions (Sangeetha et al., 2010). Phenolics are known to have fungitoxic effect and a role in strengthening the host cell wall. PGPR treated plants showed high level accumulation of phenolic compounds when challenged with pathogens (M'Piga et al., 1997). Similarly, pre-treatment with PGPR had shown induction of phenolics against *F. oxysporum* f. sp. *pisi* and *Pythium ultimum* in pea and cucumber, respectively (Benhamou et al., 1996, 2000). In the present study, among the different defense related enzymes, PPO was found to be induced significantly in BS2 treated plants challenged with FOL. Similarly in native PAGE, induction of new or enhanced accumulation of PPO in tomato pretreated with BS2 against FOL indicated that PPO also played a crucial role

in protecting the crop from the soil borne pathogen. Ramamoorthy et al. (2002) also noted unique induction of PPO in native PAGE analysis in PGPR treated tomato and chilli against *P. aphanidermatum*.

In conclusion, induction of defense related enzymes and phenolics by *Bacillus* PGPR, was found to have protective role in combating the wilt disease caused by *F. oxysporum* f.sp. *lycopersici* in tomato which in turn improved the fruit yield and quality.

Conflict of interests

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

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