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Efficacy of indigenous plant growth-promoting rhizobacteria and *Trichoderma* strains in eliciting resistance against bacterial wilt in a tomato

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Abstract

Bacterial wilt of tomato caused by *Ralstonia solanacearum* is a serious threat to tomato production worldwide. For eco-friendly management of bacterial wilt of tomato, the rhizospheric microorganisms belonging to the genera *Bacillus* (6 isolates), *Brevibacillus* (1 isolate), *Pseudomonas* (3 isolates), and *Trichoderma* (8 isolates) were studied for their ability to induce innate immunity in tomato, individually and in combination against *R. solanacearum* in greenhouse and field studies. In laboratory studies, maximum germination percent of 93%, vigor index of 1609 was noted in seed bacterization with *P. fluorescens* Pf3, followed by 91% germination, vigor index of 1593 in treatment with *T. asperellum* T8 over control. Under greenhouse conditions, protection against bacterial wilt in individual treatments with PGPRs ranged from 38 to 43% and *Trichoderma* sp. ranged from 39 to 43% in comparison to control. In comparison to individual seed treatment, among different combinations, maximum seed germination percent of 97% was recorded with combination *P. fluorescens* Pf3 + *T. longibrachiatum* UNS11. In greenhouse studies' combination seed treatment with *P. fluorescens* Pf3 + *T. longibrachiatum* UNS11 offered an impressive 62% protection against bacterial wilt over control. Similarly, under field conditions, seed treatment with *P. fluorescens* Pf3 + *T. longibrachiatum* UNS11 resulted in 61% protection. The innate immunity triggered by eco-friendly seed treatment was analyzed by expression to defense-related enzymes such as peroxidase, phenylalanine ammonia-lyase, and polyphenol oxidase in comparison to control. This study indicated that the potential benefits of using combination treatments of beneficial microorganisms in effectively inducing resistance are possible for dual benefits of enhanced plant growth, tomato yield, and pathogen suppression.

Keywords: *Ralstonia solanacearum*, Seed treatments, Tomato, Combinations, Defense enzymes, *Trichoderma*, PGPR

Background

Tomato (*Solanum lycopersicum* L.) is one of the greatest economically significant solanaceous vegetable developed worldwide and major crop after sweet potato and potato. India positions second in tomato production worldwide (Prajapati et al. 2014). Tomato plants are

susceptible to more than 200 diseases caused by bacteria (bacterial wilt, bacterial canker, bacterial speck, and bacterial leaf spot), fungi (*Fusarium* wilt, *Verticillium* wilt, root rot, *Alternaria* stem canker, powdery mildew, etc.), and viruses (tomato mosaic virus, tomato spotted wilt virus, and tomato yellow leaf curl virus) (Nowicki et al. 2013). Among these diseases, bacterial wilt incited by *Ralstonia solanacearum* is one of the devastating infections of tomato crops in the tropical and subtropical areas of the world (Wei et al. 2018). Based on the

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economic and scientific position in plant pathogens, among the top 10 bacterial pathogens, *R. solanacearum* placed as the second most bacterial pathogen that causes a vascular wilt and is one of the most damaging pathogens with rapid and fatal wilting symptoms (Mansfield et al. 2012).

R. solanacearum is the soil-borne bacterial phytopathogen, and its varied host variety causes wilt in more than 450 plant species in 54 families (Milling et al. 2011). The wilt limits the production of numerous crops such as tomato, eggplant, ginger, potato, chilli, banana, and groundnut in India (Narasimha Murthy and Srinivas 2012; Mansfield et al. 2012). The bacterial wilt has been reported mostly from tropical, subtropical, and warm temperate areas including Karnataka, Andhra Pradesh, Odisha, Kerala, Goa, West Bengal, Maharashtra, Assam, Jharkhand, Himachal Pradesh, Uttarakhand, etc. (Devendra et al. 2018). *R. solanacearum* grows in vascular systems of hosts that are responsible for symptoms of wilt. The presence of the *R. solanacearum* inside the xylem is multiplied with the production of extracellular polysaccharides which block the vascular vessels inducing a water shortage throughout the plant.

The control of bacterial wilt in tomato has been difficult because it has a wide host range, limited possibility for chemical control, long survival rate in soil, and genetic diversity of *R. solanacearum* (Elphinstone 2005). Presently, bactericide application is one of the greatest approaches for control of bacterial wilt and a variety of antibiotics are available (Yuliar et al. 2015). Increasing the usage of synthetic bactericides for control of wilt causes numerous detrimental effects on human health and environment (Satapute et al. 2019). Due to the hazards associated with synthetic pesticides, disease managements through biological control are the new developing knowledge and gaining significance in well farming sustainability (De-Britto et al. 2020). Biological agents, well defined as living microorganisms, can expressively decrease the plant pathogen density (O'Brien 2017). Certain bacteria and fungi are discussed as plant growth-promoting rhizobacteria (PGPR) and plant growth-promoting fungi (PGPF), respectively, and their efficacy in controlling various plant diseases affected by plant pathogens has been extensively recognized (Abdelrahman et al. 2016 and Zhang et al. 2020). Induced systemic resistance (ISR) is an improvement of plant self-protective capability against different phytopathogens (Romera et al. 2019).

Numerous beneficial microbes can induce defense reaction and decrease disease frequency in various host-pathogen interactions (Kloepper et al. 2004). These PGPRs and PGPFs trigger plant defense mechanisms against plant pathogens by improving the defense enzymes like peroxidase (POX), phenylalanine ammonia-lyase (PAL), and

polyphenol oxidase (PPO). Combined applications of two or more beneficial microbes are more active in controlling the plant diseases than using an individual microorganism (Maketon et al. 2008). The combination method had an advantage in several features to the single strains such as a wider range activity, more consistent biocontrol effectiveness, and a more defense activity (Latha et al. 2009).

Hence, the present study was conducted to analyze the ability of PGPR and *Trichoderma* strains in improving seed germination and seedling vigor under laboratory conditions and the effect of selected PGPR and *Trichoderma* strains individual and with combined seed treatment in the induction of resistance under greenhouse and field studies. The ability of strains in inducing resistance was analyzed by the activity of defense-related enzymes in treated and control seedlings.

Materials and methods

PGPR, *Trichoderma* strains, and inoculum preparation

Plant growth-promoting rhizobacterial strains (*P. fluorescens*) and *Trichoderma* sp. were isolated from rhizosphere soil on nutrient agar (NA) medium (Hi Media-Mumbai) and potato dextrose agar medium (PDA) (Hi Media-Mumbai), respectively, by serial dilution method (Narasimha Murthy et al. 2013 and McPherson et al. 2018). A total of 18 bacterial strains and *Trichoderma* sp. were used in the present study. Among the 18 bacterial strains, 6 strains of *Bacillus* sp., one strain of *Brevibacillus* sp., 3 strains of *P. fluorescens*, and 8 strains *Trichoderma* sp., 7 PGPRs, *B. amyloliquefaciens* IN937a, *B. subtilis* GB03, *B. subtilis* SE34, *B. pumilus*T4, *Brevibacillus brevis* IPC11, *B. subtilis* IN937b, and *Bacillus pumilus* INR7 were obtained from culture collections of the Department of Entomology and Plant Pathology, Auburn University, Alabama (courtesy of Professor Kloepper J.W and Professor M.S. Reddy). *Pseudomonas fluorescens* Pf3 (KF679344), *P. fluorescens* Pf5 (KF679345), *P. fluorescens* Pf8 (KF679346), *Trichoderma asperellum* T4 (KF679342), *T. asperellum* T8 (KF679343), *T. harzianum* UNS10 (MK611661), *T. harzianum* UNS35 (MK611662), *T. longibrachiatum* UNS11 (MK611663), *T. longibrachiatum* UNS11 (MK611664), *T. viride* UNS35 (MK886525), and *T. viride* UNS42 (MK886526) were isolated and molecular identification from different rhizosphere soil.

Bacterial cell suspensions were prepared by growing in 500-ml conical flasks having a 200-ml nutrient broth (NB) medium (Hi Media-Mumbai) on a shaker at 120 rpm at 28 ± 2 °C for 24 h. The cells were harvested by centrifuging at 6000 rpm for 5 min, and the bacterial pellet was re-suspended in distilled water and was adjusted to OD 0.1 at 600 nm by a UV-visible spectrophotometer (Elico-Japan) to get a 1×10^8 cfu/ml. For the preparation of *Trichoderma* sp. inoculum, a 7-day-old culture on

PDA agar (HiMedia-Mumbai) plates were harvested by the addition of 10 ml of sterile distilled water and gently brushing the culture surface by a bent sterile glass rod. The cell suspension was filtered by using double-layered cheesecloth to eliminate large mycelial remains. The concentration of spore was assessed by hemocytometer, and 5×10^9 spores/ml was used. The *Trichoderma* spores were mixed with sterilized talc powder, and the final concentration of the carrier material per gram was 5×10^9 spores per gram. After shade dry, the talc formulation was filled in a polypropylene bag and closed (Jayaraj et al. 2006).

Preparation of *R. solanacearum* inoculum

The *R. solanacearum* (RS5-KF924743) inoculum was prepared by the inoculation of a pathogen in casamino acid peptone glucose (CPG) broth (Hi Media-Mumbai) and incubated for 48 h on a rotary shaker at 150 rpm at 30 °C. The bacterial cells were harvested in sterile distilled water by centrifugation at 12,000 rpm at 10 °C for 10 min. The *R. solanacearum* cell pellet was resuspended in sterilized distilled water, bacterial concentration was adjusted to 1×10^8 cfu/ml, and absorbance was adjusted absorbance at 600 nm using UV-visible spectrophotometer (Elico-Japan) (Ran et al. 2005).

Mode of seed treatment

One gram of tomato seeds (Arka Meghali) was surface-sterilized with 10 ml of 1% (v/v) sodium hypochlorite for 3 min, washed thoroughly twice in distilled water, and shade dry on a blotter sheet. Bacterization of the surface-sterilized tomato seeds was attained by soaking in bacterial suspensions, preparation of bacterial suspensions as described earlier and added with 0.2% sterilized carboxymethyl cellulose (CMC) as a sticker. The bacterial suspensions were incubated at 26 °C in a shaker for 6 h to bind the bacterial cells to the seed coat. After incubation, the seeds were allowed to shade dry. The seeds treated with distilled water modified with CMC served as a control. Strains of *Trichoderma* sp. in talc powder formulation was prepared by aseptically mixing 400 ml of 5×10^9 spores, with 1 kg of earlier sterilized talc powder. The talc powder was autoclaved at 121 °C for 30 min on two successive days and mixed with 0.2% CMC prior to seed treatment, and the seed was mixed with the *Trichoderma* sp. formulation at 15 g/kg of seeds.

Effect of PGPR and *Trichoderma* sp. on seed germination and seedling vigor of tomato under laboratory conditions

The germination test was conducted based on the paper towel method using seeds treated with pure PGPR suspensions and *Trichoderma* sp. spores as described earlier (ISTA 2003). Treated and control seeds were seeded onto paper towels rinsed in a sterilized distilled water.

One hundred tomato seeds were positioned equidistantly on a paper towel and enclosed with another pre-soaked paper towel, rolled along with the polythene packaging to avoid drying of towels. The rolled paper towels were then incubated in an incubation chamber at 24 ± 1 °C. After incubation, the paper towels were opened and the number of germinated seeds was recorded and signified as the percent. The seedling vigor index was calculated after 10 days of incubation (Abdul Baki and Anderson 1973). To evaluate the vigor index, the mean length of the root and shoot in each variant of inoculation were measured. The vigor index (VI) was calculated using the formula $VI = (\text{mean root length} + \text{mean shoot length}) \times \text{germination percent}$. The experimentation was conducted with four replicates of hundred seeds each, and the entire experiment was repeated thrice.

Screening of PGPR and *Trichoderma* sp. against bacterial wilt disease under greenhouse conditions

In greenhouse conditions, the treated seeds were planted in sterilized plastic pots (25-cm diameter) with soil mixtures (soil: sand: farmyard in the ratio of 1:1:1). The pots were kept under greenhouse conditions such as day and night cycles of 16 and 8 h and 28 and 30 °C and 65% relative humidity. Each treatment contained five replicates, with 20 seedlings per replicate. In the seedling stage, 3 seedlings were kept in each pot. The 20-day-old plant root system of each plant was wounded with a scalpel, and the seedlings were challenge-inoculated by soil drenching at 30 ml of 1×10^8 cfu/ml of *R. solanacearum* suspension into the wounded root system of each pot (Tans-Kersten et al. 2001). Seed treatment with distilled water served as a control. Treated plants were observed for bacterial wilt indications after challenge inoculation, and wilt incidence was documented up to 30 days. Wilting incidence (%) was calculated, following the formula: $WI\% = (Nw/Nt) \times 100$, where Nw = number of wilted plants and Nt = total number of plants.

Effect of combination seed treatment with PGPR and *Trichoderma* sp. on seed germination and vigor of tomato seedling

Based on seed germination, seedling vigor and greenhouse studies, the potential 7 isolates were selected for combination study (3 PGPR strains and 4 strains of *Trichoderma* sp.) viz., *B. subtilis* SE34, *B. amyloliquefaciens* IN937a, *P. fluorescens* Pf3, *T. asperellum* T8, *T. harzianum* UNS35, *T. longibrachiatum* UNS11, and *T. viride* UNS42. The seed germination test was carried out based on the paper towel method; seeds treated with selected both bacterial suspensions and *Trichoderma* sp. spores in 4 replicates of 100 seeds each (ISTA, 2003). The germination test was performed as defined earlier.

Effect of combination seed treatment with PGPR and *Trichoderma* sp. on bacterial wilt under greenhouse conditions

The combination treatments were similar as designated above. In greenhouse experiments, the treated seedlings were transplanted in sterilized plastic pots (25-cm diameter) with soil mixtures (soil: sand: farmyard in the ratio of 1:1:1). Each treatment contained 5 replicates, with 20 seedlings per replicate, with three repeated experiments. In the seedling stage, only 3 plants were kept in each pot. Twenty-day-old seedlings were challenge-inoculated with 30 ml of *R. solanacearum* suspension by soil drenching into every pot. Treated seeds with distilled water served as control. Plants were observed for bacterial wilt symptoms after challenge inoculation, and wilt incidence was recorded up to 30 days.

Effect of combination seed treatment with PGPR and *Trichoderma* sp. on bacterial wilt incidence under field conditions

The field experiment was carried out at farming plots located in Bhoomishettihalli (BH) (13° 28' 05.7" N, 78° 04' 57.7" E), Karnataka, India, during tomato growing season of March to June in 2018. The treatments and controls were the same as those given above. Twenty-day-old-treated tomato seedlings were uprooted from portraits and transplanted to trial plots with a spacing of 60 × 45 cm. The selected individual experimental plot size was of 25 m² (Narasimha Murthy et al. 2016). Two weeks after transplantation, challenge inoculation with *R. solanacearum* suspension (5 ml/plant) by soil drenching method. Each treatment has 4 replications, and each repetition contained 100 seedlings. All the cultural procedures, application of fertilizers, etc., were followed as per the package of practices. The percentage of bacterial wilt frequency was recorded 1 week to 45 days after pathogen inoculation. Tomato fruits were periodically harvested and evaluated at average fruit weight and tomato yield per plot was transformed to kg/m² (Wydra and Semrau 2005).

Sample collection for enzyme analysis

The leaf samples of single- and combined-treated plants were sampled at 0, 6, 12, 24, 36, 48, 60, 72, 84, and 96 h after challenge inoculation with *R. solanacearum* and kept at - 80 °C until used for the following study. The treatments were included under the greenhouse: (1) inoculated control (IC) (inoculated with *R. solanacearum*), (2) un-inoculated control (UC), and (3) seedlings grown from seed treated with individual and combination of PGPR and *Trichoderma* sp. One gram of leaves of tomato was homogenized with 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 4 °C in mortar and pestle, and the homogenate was centrifuged at 12,000 rpm for 20

min. The homogenate supernatant was used as a crude extract for enzymes analysis of peroxidase (POX) (EC 1.1.1.7) and polyphenol oxidase (PPO) (E.C. 1.14.18.1) (Narasimha Murthy et al. 2016). The POX and PPO enzyme activities were expressed as changes in the absorbance min⁻¹ mg⁻¹ of fresh tissue. Protein estimations of extracts were carried out by Lowry's method using bovine serum albumin as a standard (Lowry 1951).

Statistical analyses

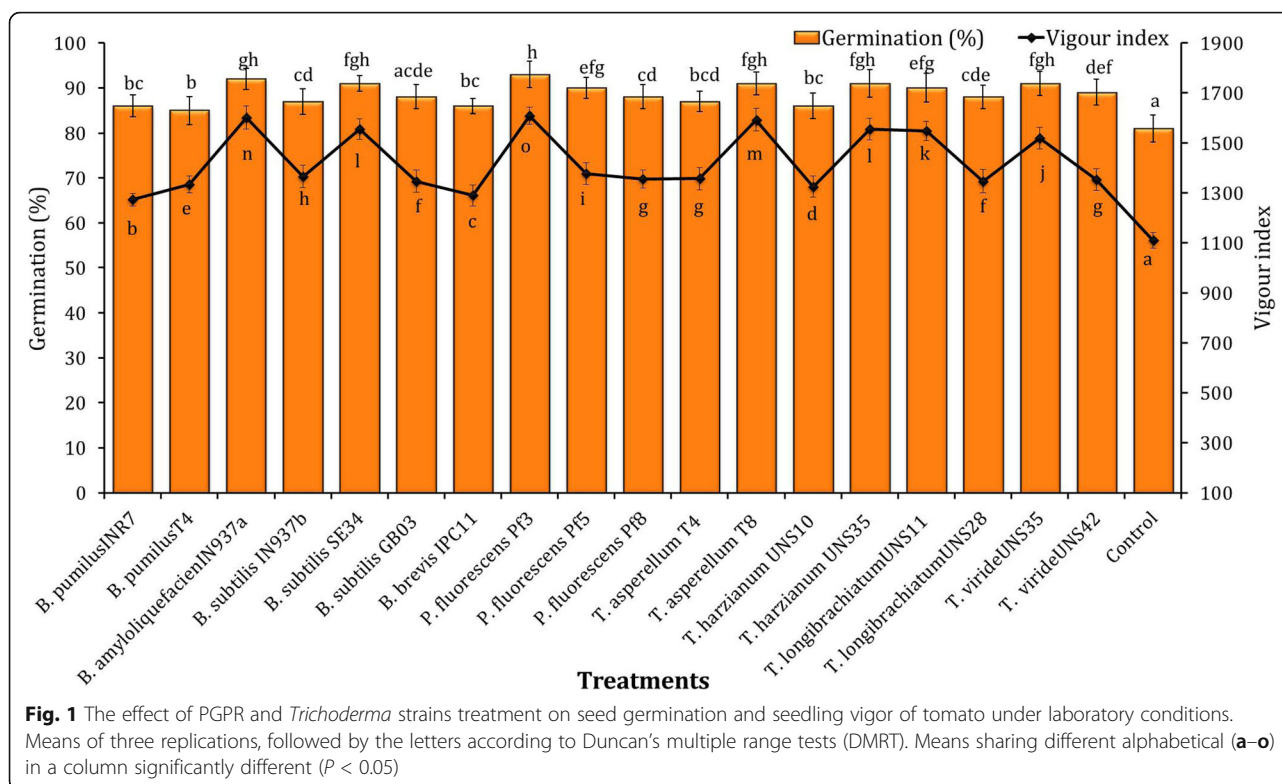
The experiments were carried out in triplicates and results were calculated as mean ± standard deviation. The analysis was performed with ANOVA and Duncan multiple range test (DMRT) test was done using SPSS software (version 20.0). The differences were measured as important when $p \leq 0.05$.

Results and discussion

Effect of PGPR and *Trichoderma* sp. on seed germination and seedling vigor of tomato under laboratory conditions

The results of the percent of seed germination revealed that all the seed treatments had improved seed germination and seedling vigor than the control. The germination percent of tomato seeds treated with PGPR strains and treated *Trichoderma* sp. ranged from 93 to 81% and 91 to 86%, respectively. Among PGPR treatments, *P. fluorescens* Pf3 showed maximum germination percent (93%), followed by *B. amyloliquefaciens* IN937a (92%), *B. subtilis* SE34 (91%), *P. fluorescens* Pf5 (90%), *B. subtilis* GB03 and *P. fluorescens* Pf8 (88%), *B. subtilis* IN937b (87%), *Bacillus pumilus* INR7 and *Brevibacillus brevis* IPC11 (86%), and *B. pumilus* T4 (85%) germination as compared to control (81%) germination (Fig. 1). Among *Trichoderma* sp. treatments, maximum germination percent of 91% was observed in with *Trichoderma asperellum* T8, *T. viride* UNS35, and *T. harzianum* UNS35, followed by *T. longibrachiatum* UNS11 (90%), *T. viride* UNS42 (89%), *T. longibrachiatum* UNS28 (88%), *T. asperellum* T4 (87%), and *T. harzianum* UNS10 (86%) as compared to control (81%). The vigor index of tomato seedlings ranged from 1609 to 1273 with PGPR treatments and with *Trichoderma* sp. ranged from 1593 to 1324 vigor indexes as compared to control (1110). Among 10 PGPR strains, the maximum seedling vigor index was recorded in seed bacterization by *P. fluorescens* Pf3 (1608.9) and among 8 *Trichoderma* sp., highest vigor index was recorded by *T. asperellum* T8 (1592.5) (Fig. 1).

In the present study, seed treatments with various PGPRs and *Trichoderma* isolates, either individually or combined, significantly improved germination and vigor index in comparison to control. The above outcomes might be due to the improved production of plant hormones such as gibberellins would have elicited the



action of specific enzymes that helped initial germination, like amylase has supported an increase in accessibility of starch assimilation. These research outcomes are in promise with Almaghrabi et al. (2013) which improved plant growth, shoot weight, root weight, plant height, number of fruits per plants, and weight of yield per plant by treatment of *P. putida*, *P. fluorescence*, *S. marcescens*, *B. amyloliquefaciens*, *B. subtilis*, and *B. cereus*. Similar studies have described improved plant growth and development in inoculated with PGPR (Van-Loon 2007; Boudyach et al. 2010).

Screening of PGPR and *Trichoderma* sp. against bacterial wilt disease under the greenhouse conditions

Among PGPR and *Trichoderma* strains assessed individually for their efficiency to induce resistance against bacterial wilt disease incidence, varied degrees of protection, ranging 38–43% under greenhouse conditions. Bacterial wilt disease incidence of 54% (43% protection) was resulted by treated with *P. fluorescens* Pf3 and *B. amyloliquefaciens* IN937a in comparison with non-bacterized control (97% bacterial wilt incidence) (Table 1). Similarly, seed treatment with *T. asperellum* T8, *T. harzianum* UNS35, and *T. viride* UNS42 resulted in 54, 54, and 55% bacterial wilt disease incidence, respectively, and disease protection resulted as 43, 42, and 42%, respectively, in comparison to control (97%) (Table 1). The soil application of *B. amyloliquefaciens* (S13-3)

inhibited bacterial wilt in tomato overproduction of antibiotics increased by induction of systemic resistance (Shoko et al. 2014).

B. amyloliquefaciens strains (CM-2 and T-5) were induced with many defense responses in a tomato plant, resulting in the control of bacterial wilt (Tan et al. 2013). The treatment of roots of tomato seedlings with *B. thuringiensis* isolate, followed by the challenged with *R. solanacearum* inhibited the wilt symptom development and induction of defense system in tomato, counting the gene expression of pathogenesis-related proteins (Hyakumachi et al. 2013).

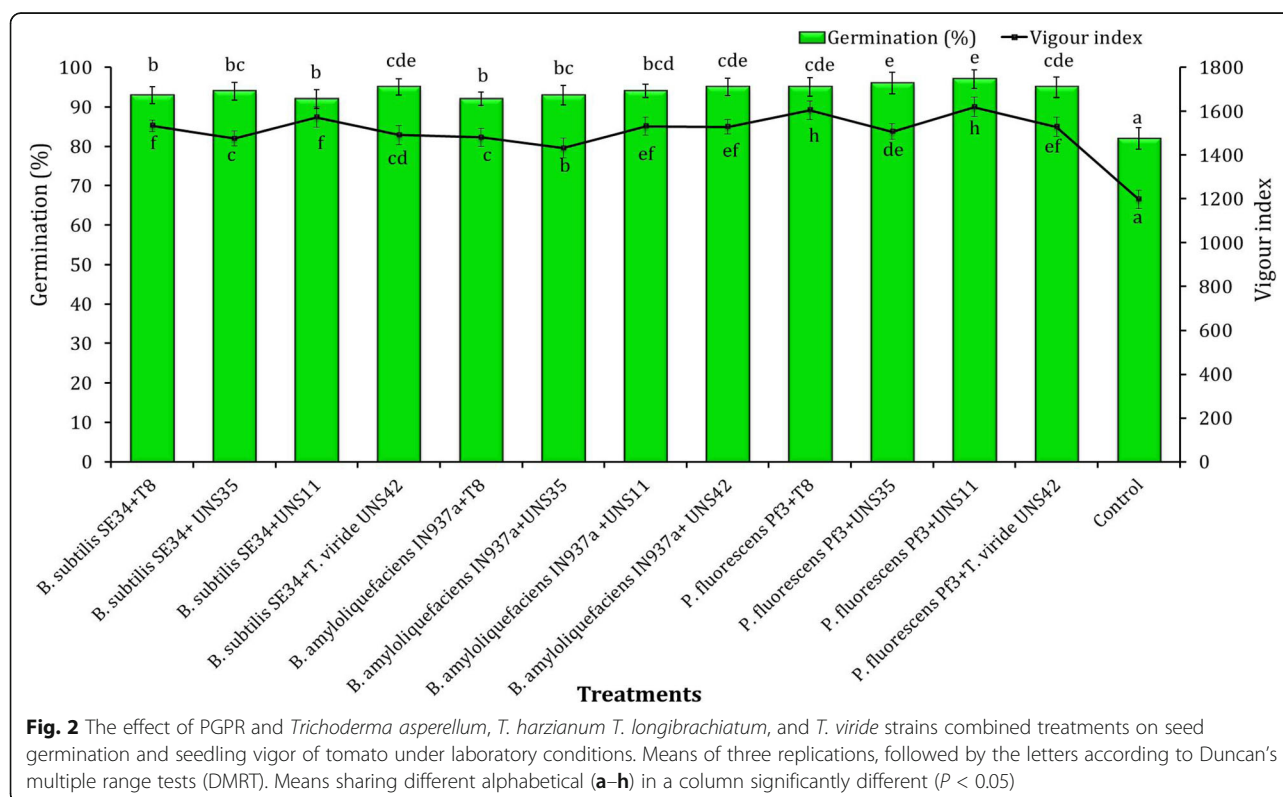
Effect of combination seed treatment with PGPR and *Trichoderma* sp. strains on seed germination and vigor of tomato seedling

The combination of PGPR and fungal strain-treated seeds revealed all the treatments showed improved seed germination percentage, vigor index as compared to control (Fig. 2). The seed germination percent and vigor index presented by a combination of PGPR and *Trichoderma* strains were greater in comparison to strain-treated alone and control (82%). Among combination seed treatment, the highest seed germination percent of 97% was recorded in treatment with *P. fluorescens* Pf3 + *T. longibrachiatum* UNS11 combination, followed by 96% with *P. fluorescens* Pf3 + *T. harzianum* UNS35. Treatment with *B. subtilis* SE34 + *T. viride* UNS42, *P.*

Table 1 Screening of PGPR and *Trichoderma* strains for their potential to induce resistance against bacterial wilt under greenhouse conditions

Treatments	Disease incidence (%)	Disease protection (%)
<i>Bacillus pumilus</i> INR7	59.6 ^k	37.8 ^b
<i>B. pumilus</i> T4	57.6 ^{fgh}	39.8 ^{cde}
<i>B. amyloliquefaciens</i> IN937a	54.6 ^{abc}	42.8 ^{hij}
<i>B. subtilis</i> IN937b	58.7 ^{ijk}	38.7 ^{bc}
<i>B. subtilis</i> SE34	56.4 ^{de}	41.0 ^{fg}
<i>B. subtilis</i> GB03	57.5 ^{fg}	39.9 ^{de}
<i>Brevibacillus brevis</i> IPC11	59.3 ^{jk}	38.1 ^b
<i>P. fluorescens</i> Pf3	54.3 ^{ab}	43.1 ^{ij}
<i>P. fluorescens</i> Pf5	58.6 ^{hijk}	38.8 ^{bcd}
<i>P. fluorescens</i> Pf8	57.7 ^{fghi}	39.7 ^{cde}
<i>T. asperellum</i> T4	58.4 ^{ghij}	39.0 ^{cde}
<i>T. asperellum</i> T8	54.1 ^a	43.3 ^j
<i>T. harzianum</i> UNS10	57.2 ^{ef}	40.2 ^{ef}
<i>T. harzianum</i> UNS35	54.8 ^{abc}	42.6 ^{hij}
<i>T. longibrachiatum</i> UNS11	55.6 ^{cd}	41.8 ^{gh}
<i>T. longibrachiatum</i> UNS28	57.4 ^{efg}	40.0 ^e
<i>T. viride</i> UNS35	57.6 ^{fgh}	39.8 ^{cde}
<i>T. viride</i> UNS42	55.2 ^{bc}	42.2 ^{hi}
Control	97.4 ^l	0.0 ^a

Means of three replications, followed by the letters according to Duncan's multiple range tests (DMRT). Means sharing different alphabetical (a–k) superscripts in a column significantly different ($P < 0.05$). Control *R. solanacearum*-treated plants



fluorescens Pf3 + *T. asperellum* T8, and *P. fluorescens* Pf3 + *T. viride* UNS42 resulted in seed germination of 95% with comparison to control. Treatment with *B. subtilis* SE34 + *T. harzianum* UNS35 and *B. amyloliquefaciens* IN937a + *T. longibrachiatum* UNS11 resulted in seed germination of 94%. Similarly, treatment with *B. subtilis* SE34 + *T. asperellum* T8 and *B. amyloliquefaciens* IN937a + *T. harzianum* UNS35 resulted in 93% germination. Combination treatments with *B. subtilis* SE34 + *T. longibrachiatum* UNS11 and *B. amyloliquefaciens* IN937a + *T. asperellum* T8 resulted in 92% seed germination in comparison to control (82%) (Fig. 2). Seed treatments with a combination of strains improved vigor index in comparison to un-inoculated control (1197.2). Maximum vigor index of 1605.5 and 1619.9 was recorded in *P. fluorescens* Pf3 + *T. asperellum* T8 and *P. fluorescens* Pf3 + *T. longibrachiatum* UNS11, respectively (Fig. 2). Present results agreed with the Abo-Elyousr et al. (2019) reported that seed primed with *B. subtilis*, *B. amyloliquefaciens*, *P. fluorescens*, and *P. aeruginosa* increased the seed germination up to 90.0%.

Effect of seed treatment combination with PGPR and *Trichoderma* sp. on bacterial wilt under the greenhouse conditions

Complete valuation of wilt protection presented by the combination of both PGPR and *Trichoderma* sp. were

significantly higher than wilt protection presented by strains treated individually (Fig. 3). A combination of *P. fluorescens* Pf3 + *T. longibrachiatum* UNS11 was found wilt protection of 62% as compared to control and other combinations (Table 2).

In the present study, results were found that a combination of PGPR and *Trichoderma* strains had higher disease control than those of single-treated strains. A total of 12 consortia of PGPR and *Trichoderma* sp. were studied under the greenhouse and field conditions against bacterial wilt of tomato. The combined applications of PGPR and *Trichoderma* strains have reported to be effective under field conditions against bacterial wilt of tomato. Field trial outcomes evidently confirm that the treatment of PGPR and *Trichoderma* sp. as potential inducers in inducing resistance in tomato plants against bacterial wilt. The treatments of *P. fluorescens* Pf3 + *T. longibrachiatum* UNS11 were recorded highest wilt protection of 62% as compared to control treatment (97%). Our research outcomes authenticate earlier reports that in the control of bacterial wilt, the use of beneficial microbes in combination treatments was more effective than individual agents (Thilagavathi et al. 2007 and Elsayed et al. 2020). The present results agreed with the *R. solanacearum* wilt disease reduction in tomato plants by treatment with *Bacillus* sp. and *P. fluorescens* described by Guo et al. (2004). Rahman et al. (2018)

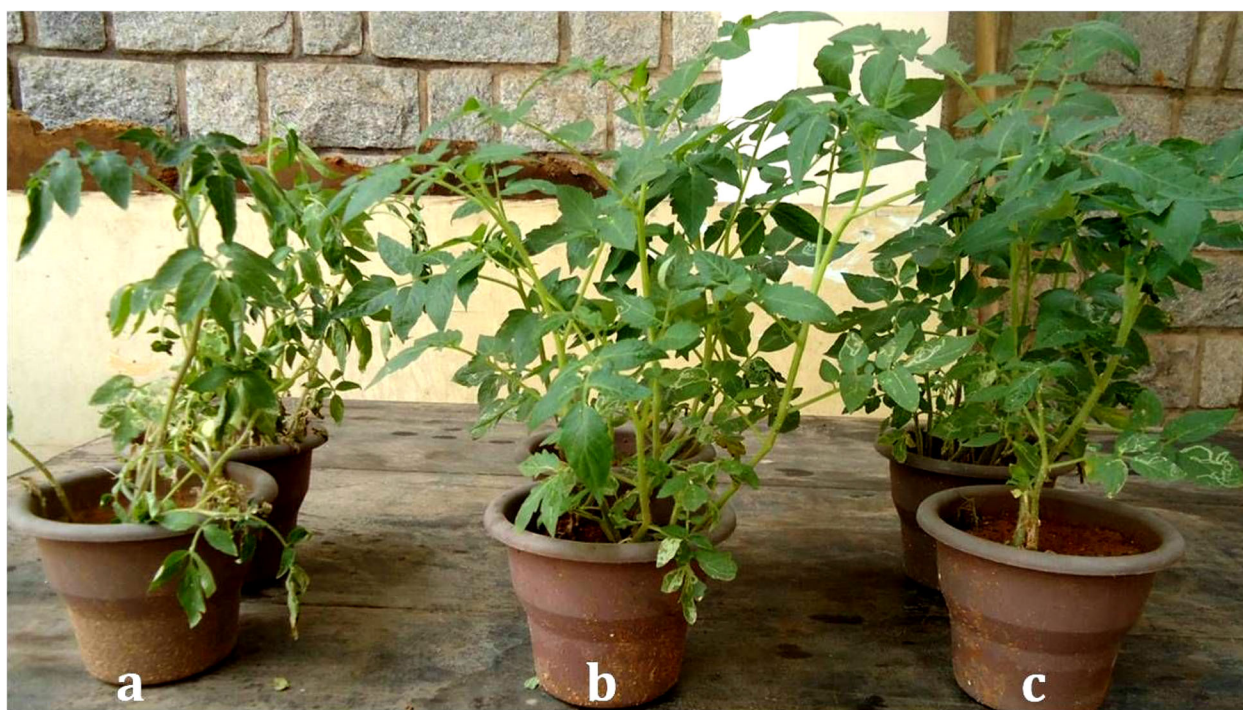


Fig. 3 The effect of combined seed treatment with PGPR and *Trichoderma* strains on the induction of resistance against bacterial wilt in tomato under greenhouse conditions. **a** Control. **b** Combined treatment with *B. amyloliquefaciens* IN937a+ *T. longibrachiatum* UNS11. **c** Combined treatment with *P. fluorescens* Pf3+ *T. longibrachiatum* UNS11

Table 2 Effect of seed treatment with a combination of PGPR and *Trichoderma* strains on bacterial wilt incidence under greenhouse conditions

Treatments	Disease incidence (%)	Disease protection (%)
Control	98.1 ^m	0.0 ^a
<i>B. subtilis</i> SE34 + <i>T. asperellum</i> T8	40.3 ^{def}	57.8 ^{hij}
<i>B. subtilis</i> SE34 + <i>T. harzianum</i> UNS35	41.2 ^{efgh}	56.9 ^{fghi}
<i>B. subtilis</i> SE34 + <i>T. longibrachiatum</i> UNS11	42.6 ^{kl}	55.5 ^{bcd}
<i>B. subtilis</i> SE34 + <i>T. viride</i> UNS42	41.4 ^{ghij}	56.7 ^{defg}
<i>B. amyloliquefaciens</i> IN937a + <i>T. asperellum</i> T8	41.3 ^{fghi}	56.8 ^{efgh}
<i>B. amyloliquefaciens</i> IN937a + <i>T. harzianum</i> UNS35	43.2 ^l	54.9 ^b
<i>B. amyloliquefaciens</i> IN937a + <i>T. longibrachiatum</i> UNS11	40.2 ^{de}	57.9 ^{hij}
<i>B. amyloliquefaciens</i> IN937a + <i>T. viride</i> UNS42	41.8 ^{ghijk}	56.3 ^{cdefg}
<i>P. fluorescens</i> Pf3 + <i>T. asperellum</i> T8	38.3 ^{bc}	59.8 ^{kl}
<i>P. fluorescens</i> Pf3 + <i>T. harzianum</i> UNS35	38.6 ^{bc}	59.5 ^{kl}
<i>P. fluorescens</i> Pf3 + <i>T. longibrachiatum</i> UNS11	36.2 ^a	61.9 ^m
<i>P. fluorescens</i> Pf3 + <i>T. viride</i> UNS42	38.8 ^c	59.3 ^k

Means of three replications, followed by the letters according to Duncan's multiple range tests (DMRT). Means sharing different alphabetical (a–m) superscripts in a column significantly different ($P < 0.05$). Control - *R. solanacearum*-treated plants

described that the *B. amyloliquefaciens* to significantly improve growth, disease suppression, and to elicit plant innate immunity. Three strains of PGPR, *B. subtilis* MBI600, GBO3, and *Bacillus amyloliquefaciens* IN937 have been reported as inducers against several plant pathogens in numerous field and vegetable crops (Zehnder et al. 2000; Beneduzi et al. 2012). The combined treatments of *Pseudomonas* sp., *Bacillus* sp., and *T. harzianum* were improved disease inhibition than their single treatment against bacterial wilt (Liu et al. 2014; Yendyo et al. 2017).

Effect of seed treatment with a combination of PGPR and *Trichoderma* sp. on bacterial wilt incidence under field conditions

Under field conditions, the outcomes showed that all the combined treatments offered maximum bacterial wilt protection over control. The percentage of wilt protection was recorded in combination with PGPR and *Trichoderma* sp. treatments ranged from 54 to 62%. Among the combination treatments, *P. fluorescens* Pf3 + *T. longibrachiatum* UNS11 exhibited maximum wilt protection of 62% than the control (97% wilt incidence) (Table 3).

Table 3 Effect of seed treatment with combination of PGPR and *Trichoderma* strains on bacterial wilt incidence under field conditions

Treatments	Disease incidence (%)	Disease protection (%)	Yield kg/m ²
Control	96.8 ^l	0.00 ^a	41.24 ^a
<i>B. subtilis</i> SE34 + <i>T. asperellum</i> T8	42.4 ^{hij}	54.4 ^{cd}	129.32 ^{de}
<i>B. subtilis</i> SE34 + <i>T. harzianum</i> UNS35	40.2 ^{de}	56.6 ^{fghi}	131.66 ^{ef}
<i>B. subtilis</i> SE34 + <i>T. longibrachiatum</i> UNS11	41.9 ^{ghij}	54.9 ^{cdef}	127.76 ^{bc}
<i>B. subtilis</i> SE34 + <i>T. viride</i> UNS42	40.5 ^{def}	56.3 ^{fghi}	132.84 ^{fg}
<i>B. amyloliquefaciens</i> IN937a + <i>T. asperellum</i> T8	41.4 ^{fghi}	55.4 ^{defg}	128.42 ^{de}
<i>B. amyloliquefaciens</i> IN937a + <i>T. harzianum</i> UNS35	42.5 ^{jk}	54.3 ^{bc}	131.73 ^g
<i>B. amyloliquefaciens</i> IN937a + <i>T. longibrachiatum</i> UNS11	40.4 ^{cdef}	56.4 ^{fghi}	128.41 ^{de}
<i>B. amyloliquefaciens</i> IN937a + <i>T. viride</i> UNS42	41.1 ^{efg}	55.7 ^{fgh}	131.36 ^g
<i>P. fluorescens</i> Pf3 + <i>T. asperellum</i> T8	37.9 ^b	58.9 ^k	130.67 ^{ef}
<i>P. fluorescens</i> Pf3 + <i>T. harzianum</i> UNS35	38.4 ^b	58.4 ^k	131.81 ^{fg}
<i>P. fluorescens</i> Pf3 + <i>T. longibrachiatum</i> UNS11	35.2 ^a	61.6 ^l	137.83 ^{gh}
<i>P. fluorescens</i> Pf3 + <i>T. viride</i> UNS42	38.7 ^{bc}	58.1 ^{ijk}	130.35 ^{ef}

Means of three replications, followed by the letters according to Duncan's multiple range tests (DMRT). Means sharing different alphabetical (a–l) superscripts in a column significantly different ($P < 0.05$). Control - *R. solanacearum*-treated plants

Outcomes of the study showed that the combination of treatments of PGPR strains and *Trichoderma* sp. significantly increased the tomato yield. Maximum tomato yield was observed in combined treatment with *P. fluorescens* Pf3 + *T. longibrachiatum* UNS11 by 137.83 kg/m² as compared to other combinations and pathogen treatment (41.24 kg/m²). The yields of tomato, treatment with *P. fluorescens* Pf3 + *T. longibrachiatum* UNS11, were significantly increased by 70% as compared to the pathogen treatment (Table 3).

Earlier reports showed that combinations were more active than single treatment of whichever bacteria or fungi (Zheng et al. 2019). Recently, there are several root-associated beneficial microorganisms have been used to activate resistance against bacterial wilt in tomato (Kurabachew et al. 2013). Pre-treatment with several biotic and abiotic inducers induce plant defense response against pathogen attack in plants against plant viruses (Udayashankar et al. 2012), fungi (Jogaiah et al. 2018), and bacteria (Narasimha Murthy et al. 2018).

Enzyme extraction and assay

The PGPR and *Trichoderma* sp. influenced the changes in defense enzymes and the highest activities of POX and PPO enzymes occurred in different periods (Figs. 4, 5, 6, and 7). The study of wilt resistance in tomato seedlings combined treatments showed the maximum activity and expression of defense-related proteins against *R. solanacearum*. Treated tomato seedlings exhibited the

expression of POX and PPO after post inoculation of *R. solanacearum*. A significantly higher POX and PPO enzyme activities were observed in combined treated with PGPR and *Trichoderma* strains when compared to single or individual treatments. The POX and PPO activities reached the maximum in all treatments at 36 h and 48 h, respectively, after *R. solanacearum* inoculation and then slowly reduced. The POX and PPO enzyme activities were higher when combined with PGPR and *Trichoderma* strains, whereas no variation witnessed in untreated control seedlings (Figs. 4, 5, 6, and 7).

The treatment with beneficial microbes induces systemic resistance against bacterial wilt has already been reported by Jogaiah et al. (2013). The better induction of defense enzymes has been recommended as a mechanism accountable for the improved by the combination of beneficial microorganisms against bacterial wilt in tomato plants (Jetiyanon et al. 2007; Hyakumachi et al. 2013; Villena et al. 2018; Zheng et al. 2019). Increased activity of ISR induced by combination treatments may be due to the increased number of beneficial microbes involved in the treatment and moreover due to the cooperation among the strains. Several reports have designated that ET, SA, and JA signaling pathways are intricate in the beneficial microbe-mediated ISR against bacterial wilt (Takahashi et al. 2014). In the present study, results presented that combined treatments of PGPR and *Trichoderma* sp. were the maximum expression of defense enzymes against bacterial wilt in tomato. The combined

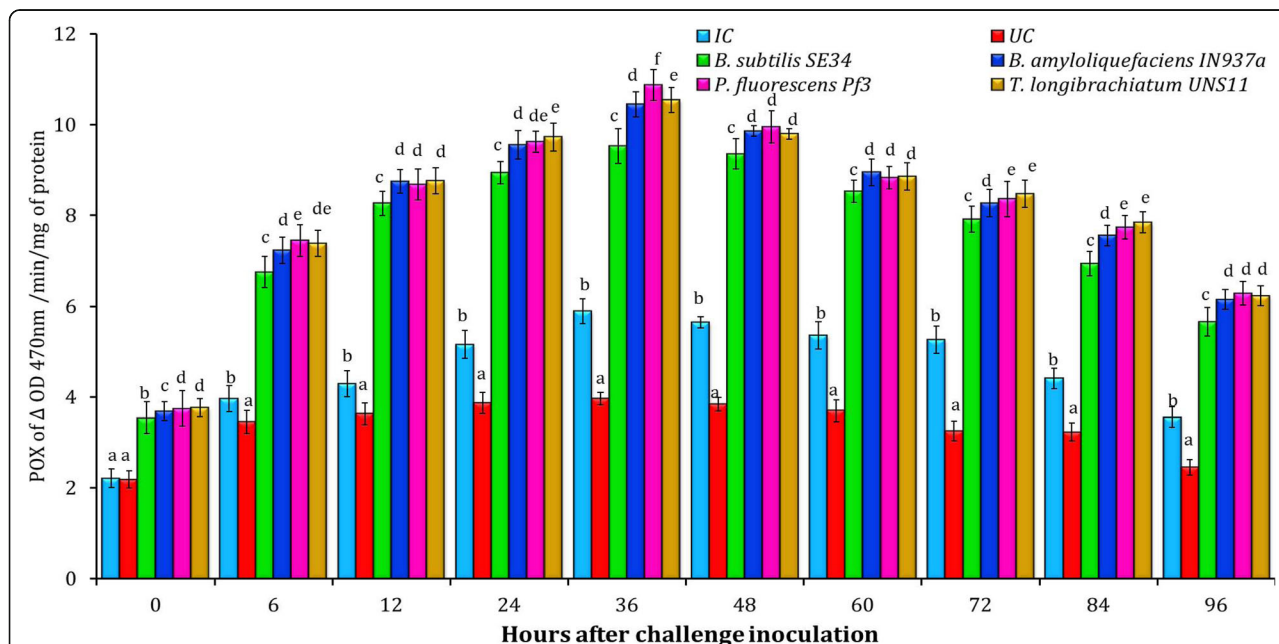


Fig. 4 The effect of PGPR and *Trichoderma* strains single or individual treatments on activity of peroxidase in tomato seedlings under greenhouse conditions. The values are the mean of three replications and bars represent standard errors. UC-uninoculated control, tomato seedlings without treatments and IC-inoculated control, tomato seedlings inoculated with *R. solanacearum*. Means of three replications, followed by the letters according to Duncan's multiple range tests (DMRT). Means sharing different alphabetical (a–f) in a column significantly different ($P < 0.05$)

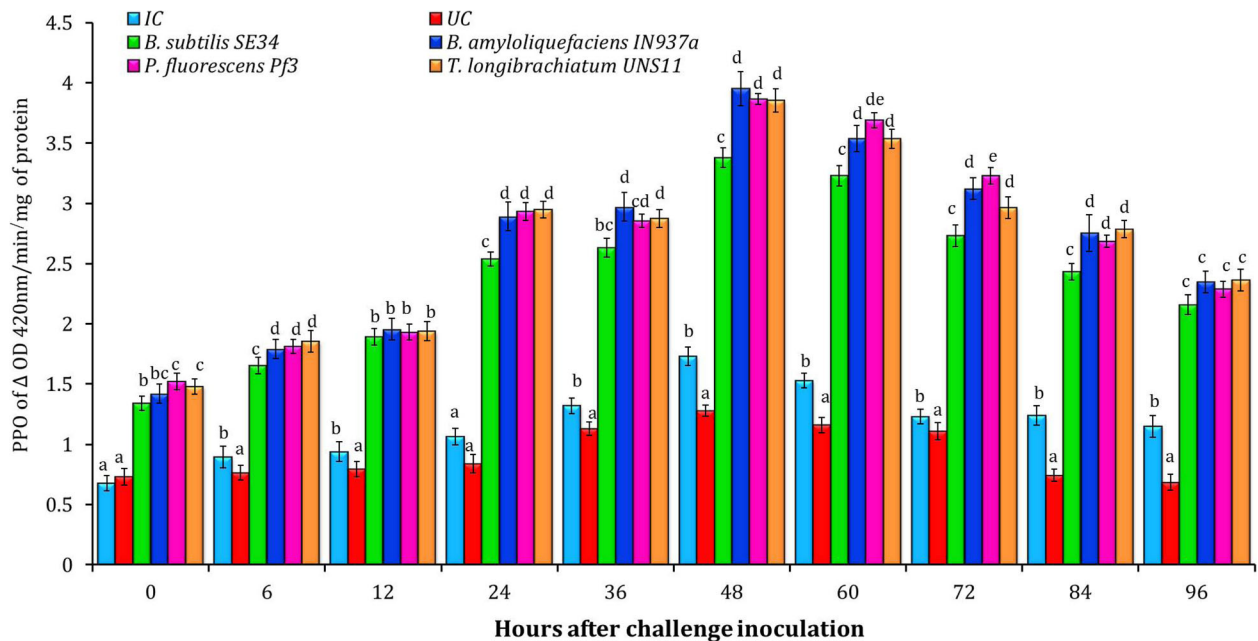


Fig. 5 The effect of PGPR and *Trichoderma* strains single or individual treatments on activity of polyphenol oxidase in tomato seedlings under greenhouse conditions. The values are the mean of three replications and bars represent standard errors. UC-uninoculated control, tomato seedlings without treatments and IC-inoculated control, tomato seedlings inoculated with *R. solanacearum*. Means of three replications, followed by the letters according to Duncan's multiple range tests (DMRT). Means sharing different alphabetical (a–d) in a column significantly different ($P < 0.05$)

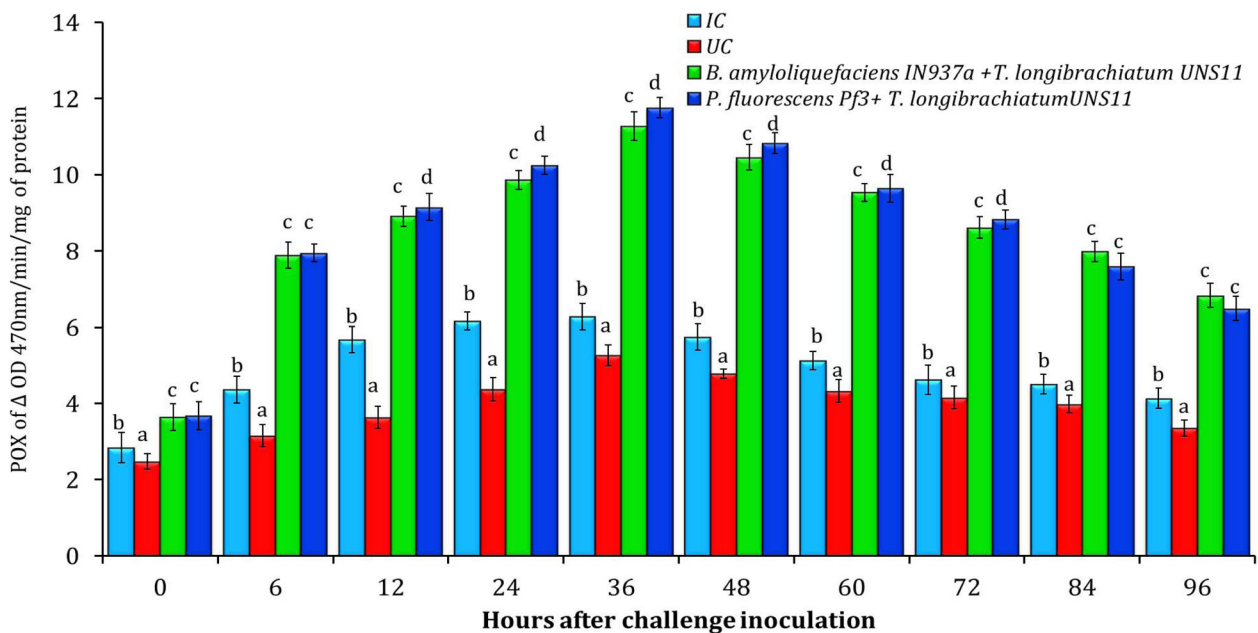
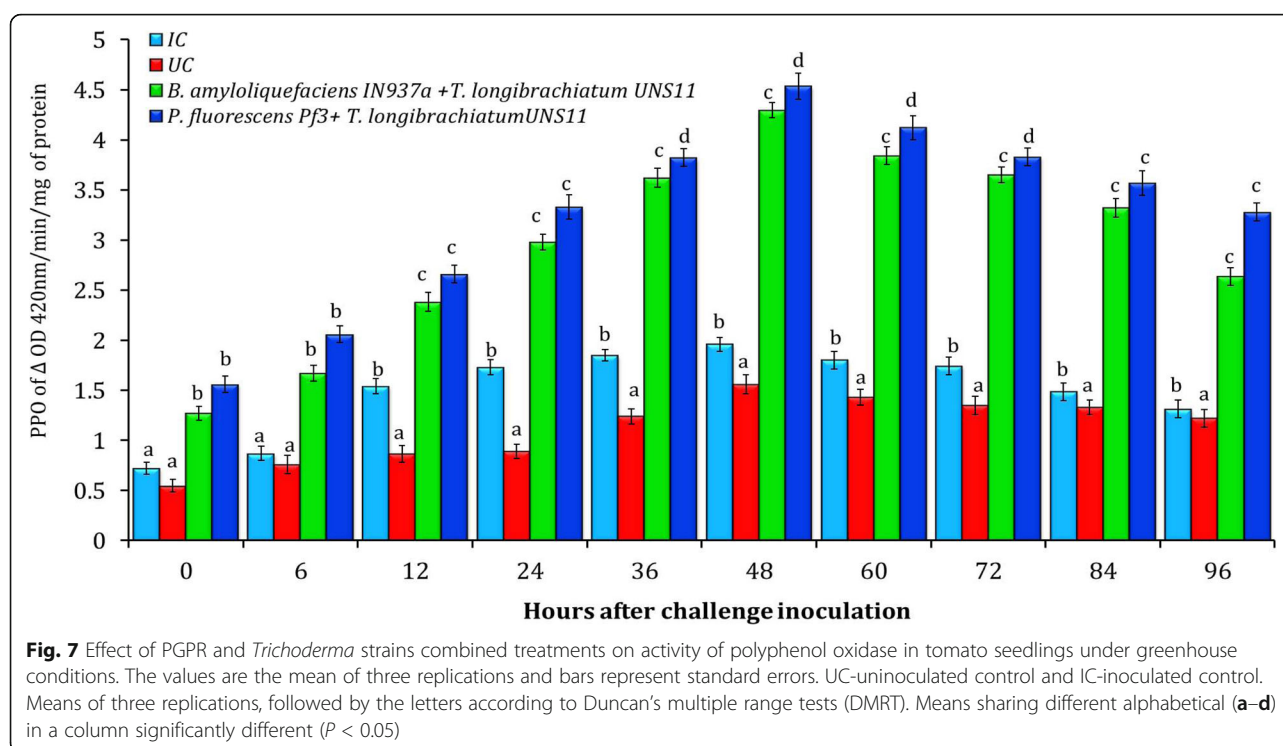


Fig. 6 The effect of PGPR and *Trichoderma* strains combined treatments on activity of peroxidase in tomato seedlings under greenhouse conditions. The values are the mean of three replications and bars represent standard errors. UC-uninoculated control, tomato seedlings without treatments and IC-inoculated control, tomato seedlings inoculated with *R. solanacearum*. Means of three replications, followed by the letters according to Duncan's multiple range tests (DMRT). Means sharing different alphabetical (a–d) in a column significantly different ($P < 0.05$)



treated tomato seedlings exhibited the expression of POX and PPO enzymes after challenge inoculation of *R. solanacearum*. A significantly higher POX and PPO enzyme activities were recorded in combined treatment with PGPR and *Trichoderma* strains when compared to single or individual treatments, whereas no variation was witnessed in untreated control seedlings. Results achieved in the present investigation on integrated control of bacterial wilt of tomato designated that all the treatments were attempted significantly enhanced the seed germination, reduced the bacterial wilt frequencies over the controls. Several beneficial microorganisms may improve the level and steadiness by providing several mechanisms of action, more steady rhizosphere community, and be active over a broader range of ecological conditions (Mostafa et al. 2016 Marian et al. 2018).

Conclusion

In the present study, *Bacillus* sp., *P. fluorescens* sp., and *Trichoderma* sp. seemed to be the best biocontrol agents in controlling bacterial wilt caused by *R. solanacearum*. A total of 18 beneficial microorganisms were found effective under in vitro and in vivo conditions, individually and in combination treatments against the disease. The present findings can benefit farmers through increasing productivity, yield and income via reducing inputs, and nonchemical means in the face of bacterial wilt epidemics. It is an evident that beneficial microbes could possibly serve as

eco-friendly and sustainable alternatives to the hazardous chemicals used for the management of plant diseases.

Abbreviations

PGPR: Plant growth-promoting rhizobacteria; PGPF: Plant growth-promoting fungi; ISR: Induced systemic resistance; SA: Salicylic acid; JA: Jasmonic acid; ET: Ethylene; POX: Peroxidase; PAL: Phenylalanine ammonia-lyase; PPO: Polyphenol oxidase; PDA: Potato dextrose agar; NA: Nutrient agar; CPG: Casamino acid peptone glucose; CMC: Carboxymethyl cellulose; VI: Vigor index; ANOVA: Analysis of variance; DMRT: Duncan's multiple range tests

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Authors' contributions

NK, SK and ACU were responsible for methodology, investigation and wrote the manuscript. CS and SRN were read the manuscript and made suitable changes. The author(s) read and approved the final manuscript.

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Availability of data and materials

All data of the study have been presented in the manuscript, and high-quality and grade materials were used in this study.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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