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Evaluation of biological efficacy of *Trichoderma asperellum* against tomato bacterial wilt caused by *Ralstonia solanacearum*

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Abstract

Bacterial wilt, caused by soilborne bacterium *Ralstonia solanacearum*, is one of the most severe diseases of tomato worldwide, and no successful control measures are available to date. In the present study, a sustainable alternative tool such as use of fungi from tomato rhizosphere is being utilized to combat the pathogen attack. The application of *Trichoderma asperellum* (T4 and T8) isolates delayed wilt development, effectively decreased the disease incidence, increased fruit yield, and improved plant growth promotion under field conditions. The *T. asperellum* treatment decreased the disease incidence by 51.06% (RS + T4) and 52.75% (RS + T8) in Bhoomishettihalli (BH) and 47.21% (RS + T4) and 46.83% (RS + T8) in Madanahalli (MH) plots, respectively when compared with the pathogen-treated plot in year 2014. Correspondent decreases in year 2015 were 50.69% (RS + T4) and 52.38% (RS + T8) in BH and 48.18% (RS + T4) and 49.22% (RS + T8) in MH plots. In year 2014, *T. asperellum* (T4 and T8) treatment enhanced the yield with 5.45 t/ha and 5.50 t/ha in BH plot and 6.66 t/ha and 6.93 t/ha in MH plot, respectively, when compared with infected plots. In year 2015, *T. asperellum* (T4 and T8) treatment enhanced the yield with 5.29 t/ha and 5.51 t/ha in BH plot and 5.82 t/ha and 5.66 t/ha in MH plot, respectively, when compared with infected plots. The disease control and yield enhancement were highest at T8, followed by T4. Increase in the level of peroxidase (POX), phenylalanine ammonium lyase (PAL), polyphenol oxidase (PPO), β-1,3-glucanase and total phenol activities at 12th, 10th, 14th, 12th, and 10th days, respectively, after pathogen inoculation was observed. This indicates the induction of plant resistance mechanism by *T. asperellum* against *R. solanacearum* in tomato plants under field conditions.

Keywords: *Ralstonia solanacearum*, Induced systemic resistance (ISR), *Trichoderma asperellum*, Tomato yield, Plant growth promoters

Background

Tomato (*Lycopersicon esculentum*) is one of the most widely cultivated vegetable crops worldwide. Vegetable crops are extremely prone to soilborne and root diseases causing huge losses in yield and its quality (Sharma et al. 2004). The main constraint to tomato production in many parts of the world is several plant diseases. Bacterial wilt is a destructive and prevalent soilborne disease that limits tomato production in the tropics, subtropics, and warm temperate regions of the world (Ramesh et al. 2014). *Ralstonia solanacearum* is one of the most severe quarantine important diseases of tomato worldwide. Its host range contains solanaceous species, leguminous species, a small number of monocotyledons, trees, shrubs, and certain ecotypes of the model plant *Arabidopsis thaliana*. The pathogen persists in soils, water, or reservoir plants for several years to form latent infections within native weeds contributing to the hard eradication of the bacterium (Avinash et al. 2016).

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Management of *R. solanacearum*, including use of resistant or tolerant varieties, cultural practices, chemical control, and biological control, are commonly employed methods to control bacterial wilt disease (Dalal et al. 1999). There are various beneficial microbes which have been completely implemented as biocontrol agents for inhibition of *R. solanacearum* under laboratory and/or greenhouse conditions, including *Pseudomonas putida*, *P. fluorescens*, *Trichoderma* spp., Bacteriophages, *Streptomyces* spp., *Acinetobacter* spp., *Enterobacter* spp., *Bacillus* spp. and *Paenibacillus macerans* (Vanitha et al. 2009 and Ling et al. 2010). *Trichoderma* isolates have strong antagonistic and mycoparasitic effects against phytopathogens and are therefore able to reduce disease severity in plants (Elsharkawy et al. 2012). The beneficial microorganisms have gained considerable attention as an ecofriendly and cost-effective platform for the stimulation of disease resistance through induced systemic resistance (ISR) and for the promotion of growth in plants for sustainable crop production (Abdelrahman et al. 2016). *Trichoderma* spp. induce plant growth by direct and indirect mechanisms (Zachow et al. 2016). *Trichoderma* spp. induce plant resistance against several phytopathogens, promote plant growth, and enhance photosynthetic activity of plants (Li et al. 2017). Presently, various reports indicate that *Trichoderma* induces systemic resistance by releasing not only proteins, but also secondary metabolites (Keswani et al. 2016).

Schonfeld et al. (2003) recorded a decrease in the *R. solanacearum* population in soil amended with decomposed organic fertilizer. The decomposed organic fertilizer or manure provides nutrients to the microbes, thus increases the biocontrol agent's ability and makes them extra competitive in the rhizosphere soil and on roots (Liu et al. 2012). Root colonization by biocontrol agents is considered a prerequisite and is directly connected to their effectiveness in controlling soilborne infections (Ji et al. 2008). The earlier studies have described that *Trichoderma* spp., *Bacillus* spp., and *Klebsiella* spp. enhanced colonize plant roots and rhizosphere, if they are applied to the soil with nutrient carrier, such as decomposed organic fertilizer or manure (Huang et al. 2011). *Trichoderma* spp. are now the greatest common fungal biocontrol agents that have been broadly studied and deployed throughout the world (Alka et al. 2017).

The objectives of the present study were to investigate biochemical responses in terms of defense enzymes and to evaluate the effectiveness of *T. asperellum* to induce systemic resistance against bacterial wilt in tomato plants, as well its effectiveness on bacterial wilt control or suppression of *Ralstonia* wilt under field conditions.

Materials and methods

Isolation and identification of microorganisms

Infected plant material and rhizosphere soil samples were collected from the wilted fields of tomato-growing areas of Karnataka. Ten virulent *R. solanacearum* strains were isolated from rhizosphere soil and shoot samples. The molecular identification of *R. solanacearum* isolates were confirmed based on 16S rRNA sequencing (Narasimha Murthy et al. 2016). *Trichoderma* spp. were isolated from healthy tomato plants' rhizosphere, using the soil dilution plate technique. Identification of *Trichoderma* spp. was further confirmed by National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute and Pune. Among ten *Trichoderma* spp. two (T4 and T8) strains were selected for field experiments based on antibacterial and greenhouse studies against *R. solanacearum* (Narasimha Murthy et al. 2013).

Preparation of bacterial inoculum

One milliliter of *R. solanacearum* stock suspension was added to casamino acid peptone glucose (CPG) broth (1-g casamino acid, 10-g peptone, 5-g glucose per liter) and incubated at 28 °C for 48 h on rotary shaker at 150 rpm (Kelman 1954). Culture broth was centrifuged at 12,000 rpm for 10 min at 10 °C. The bacterial pellet was resuspended in sterile distilled water and final concentration of suspension was set to 1×10^8 cfu/ml, by spectrophotometrically adjusting to O.D 600 nm = 0.1 (Ran et al. 2005).

Preparation of talc-based formulation

The suspension of each *Trichoderma* spp. was prepared from 7-day-old culture on potato dextrose agar (PDA), using sterile distilled water. The fungal inoculum was prepared by flooding the culture with sterile distilled water and then rubbing its surface with a bent sterile glass rod. The suspension was filtered through four layers gauze bandage to separate the spores from the mycelia. The fungal concentration in each suspension was estimated by counting with the help of hemocytometer and was adjusted to 5×10^8 spores/ml (Rojo et al. 2007). One kilogram of talc powder was taken in a sterilized metal tray, and its pH was adjusted to neutral as above. Ten grams of carboxy methyl cellulose (CMC) was added to 1 kg of talc, mixed well, and the mixture was autoclaved for 30 min at 121 °C and 15 lbs pressure, each on two consecutive days. Five hundred milliliters of spore suspension were mixed with sterilized talc powder under aseptic conditions, and the spore concentration was adjusted to 5×10^8 spores g^{-1} with a sterile talc powder. After shade drying overnight, the formulation was packed in a polypropylene bag and sealed. The formulations were mixed with 50 kg of farmyard manure and incubated for 30 days before applying to each plot.

Field experiment

Based on the previous in vitro and in vivo studies, under laboratory and greenhouse conditions (Narasimha Murthy and Srinivas 2012, and Narasimha Murthy et al. 2013), the most promising two *T. asperellum* isolates were selected for trial against the *R. solanacearum* under field conditions (Satish and Abhay 2016). The field experiment was conducted at the farmer's agricultural plots located in Bhoomishettihalli (BH) (13° 28′ 05.7″ N, 78° 04′ 57.7″ E) and Madanahalli (MH) (13° 16′ 50.7″ N, 78° 05′ 52.6″ E) near Chintamani, Karnataka, India, during tomato growing season of March–June in 2014 and 2015. The experimental fields had been selected based on cultivation of tomatoes for several years and were naturally infested with *R. solanacearum*. Seeds of wilt susceptible tomato variety Arka Meghali were procured from Indian Institute of Horticultural Research (IIHR) Bangalore, India. Four-week-old tomato seedlings were uprooted from portrays and transplanted to experimental plots and treated with *T. asperellum* (T4 and T8) farmyard manure mixture (5 g/seedling) with spacing of 60 × 90 cm. The treatments were as shown as follows: (1) control (untreated seedlings), (2) *T. asperellum* alone (T4 and T8), (3) *R. solanacearum* alone, and (4) *T. asperellum* + *R. solanacearum.* The selected individual experimental plot area was of 25 m^2 containing 14 rows with 80 or 100 plants per row, and the distance between rows was 50 cm (Narasimha Murthy et al. 2016). Buffer zones of 2 m without tomato seedlings were maintained between plots. Three replications were maintained for each treatment with 100 plants/ replication. The experiment was repeated thrice simultaneously in three different experimental plots of the fields. Seedlings were watered daily by drip irrigation, fertilized once with NPK fertilizer, farmyard manure (FYM) at 2.8 kg/m^2 and vermicompost at 0.5 kg/m^2. The NPK fertilizers consist of chemical fertilizers at the N:P:K ratio of 15:7:12; urea containing 46.5 N was applied, P applied as 7% mono superphosphate $Ca(H_2PO4)_2$, and K as potassium sulfate containing 41.7% (K_2SO_4). After 2 weeks of seedling transplantation, they were challenge inoculated by 48-h-old *R. solanacearum* suspension, 5 ml per plant by soil drenching method. The completely wilted tomato plants in each treatment were observed, 1 week after challenge inoculation up to 90 days. Disease incidence was calculated as the percentage of plants that had completely wilted. Fruits per plant, fresh weight, dry weight, plant height, stem growth, and tomato yield was calculated tons per hectare (t/ha) in each treatment, and total tomato yield was recorded at the end of the season. A total of four harvests were made at weekly intervals. The wilt incidence was evaluated when the infection emerged and calculated as the percentage of infected plants compared with the total number of growing plants in each plot. The percentage (%) of disease incidence was calculated by using the following formula: % of disease incidence = no. of wilted plants in a plot/total no. of plants in a plot × 100.

Sample collection for biochemical analysis

The leaf tissues of treated and untreated tomato plants were collected at different time intervals (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 days) after pathogen inoculation and stored in a deep freezer (− 80 °C) until used for biochemical analysis (Narasimha Murthy et al. 2016). Leaf tissues were homogenized by liquid nitrogen in a pre-chilled mortar and pestle. One gram of tomato leaf tissues was homogenized by 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 4 °C, and the homogenate was centrifuged for 20 min at 12,000 rpm. The supernatant was used as a crude extract for analyzing peroxidase (POX) (Hammerschmidt et al. 1982), polyphenol oxidase (PPO) (Mayer et al. 1965), and phenylalanine ammonia lyase (PAL) (Dickerson et al. 1984). As for the estimation of β-1,3-glucanase, 1 g of tomato leaf tissues was homogenized by 2 ml of 0.1 M sodium citrate buffer (pH 5.0) in a pre-chilled mortar and pestle, centrifuged, and supernatant was used for the estimation (Pan et al. 1991). The total phenol content was estimated as per the procedure given by Zieslin and Ben-Zaken (1993). The following treatments were included in the experiments: untreated control plants (C), plants inoculated with *R. solanacearum* alone (T1), plants inoculated with *T. asperellum* alone (T2), and plants inoculated with *T. asperellum* and challenge inoculated with *R. solanacearum* (T3).

Protein estimation

Protein estimations of all the enzyme extracts were carried out by Lowry's method (Lowry et al. 1951) using bovine serum albumin as a standard.

Native poly acrylamide gel electrophoresis analysis

The isoforms profiles of peroxidase and polyphenol oxidase were estimated by discontinuous native polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970). The protein extracts were prepared by homogenizing 1.0 g of leaf tissues with 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) and centrifuged at 20,000 rpm for 15 min at 4 °C. Samples (80-μg protein) were loaded into 8% polyacrylamide gels (Sigma, USA). After electrophoresis, POX isoforms were observed by soaking the gels in staining solution containing 0.05% benzidine (Sigma–Aldrich, Mumbai, India) and 0.03% H_2O_2 in acetate buffer (20 mM, pH 4.2) for 30 min in the dark, after which drops of 30% H_2O_2 were added with constant shaking ti1 the bands appeared (Nadolny and Sequeira 1980). For assessing the PPO isoform, the gels were equilibrated for

30 min in 0.1% 1,3-dihydroxyphenylalanine (DOPA) in 0.1 M potassium phosphate buffer (pH 7.0), followed by the addition of 10 mM catechol in the same buffer. The addition of catechol was followed by gentle shaking, resulted in appearance of dark brown discrete protein bands indicative of PPO isozymes appeared in the gel (Jayaraman et al. 1987).

Statistical analyses

All data of field trial experiments were statistically analyzed, using Microsoft Excel ™ and SPSS (version 20.0). The data were subjected to analysis of variance (ANOVA), and the means were analyzed, using Duncan's new multiple range post test at $p \leq 0.05$.

Results and discussion

Field experiment for assessment of *T. asperellum* isolates to control of bacterial wilt

The characterization of *T. asperellum* antagonism against *R. solanacearum* was the initial step in assessing the biocontrol capacity of these agents. In a previous study, *Trichoderma* spp. were screened for antagonistic activity, seed germination, and plant growth promotion under greenhouse condition against *R. solanacearum* (Narasimha Murthy et al. 2013). In the present investigation, the two potential *T. asperellum* isolates, from the rhizosphere soil of tomato plants as biocontrol agents to control *R. solanacearum*, were assessed. Soil treatment with *T. asperellum* reduced the wilt disease incidence than the untreated control and also significantly enhanced the plant growth and tomato yield (Tables 1 and 2). The plant growth was assessed, as a total amount of fruits per plant, fresh weight, dry weight, plant height, stem growth, and tomato yield. In year 2014, the disease incidence in untreated control plots was nil in both plots. In *R. solanacearum*-infected BH and MH plots, the disease incidence ranged from 85.59 to 91.07% and 84.54 to 89.65%, respectively. *T. asperellum*-treated plots showed an average of 36.43 and 40.08% disease incidence in BH and MH plots, respectively. The *T. asperellum* treatment decreased the disease incidence by 51.06% (RS + T4) and 52.75% (RS + T8) in BH and 47.21% (RS + T4) and 46.83% (RS + T8) in MH plots, respectively. The tomato yield in untreated control plot of BH was 7.52 t/ha and 7.12 t/ha in MH plot. The tomato yield in *R. solanacearum*-infected BH plot was about 1.29 to 1.69 t/ha and, in MH plot, 1.23 to 1.76 t/ha. *T. asperellum* isolate (T4 and T8)-treated plots yielded an average of 8.15 and 8.26 t/ha in BH and of 7.89 and 8.16 t/ha of tomatoes in MH, respectively. Thus, tomato yield increased by an average of 8.38 and 9.84% in BH and 10.82 and 14.61% in MH for T4 and T8 treatments, respectively, as compared with the untreated control plots (Fig. 1). Plots challenge inoculated with *R. solanacearum* and treated with T4 and T8 yielded an average of 6.27 and 6.36 t/ha in BH and 6.63 and 6.49 t/ha in MH plots, respectively. *T. asperellum* (T4 and T8) treatment enhanced the tomato yield by 5.45 t/ha and 5.50 t/ha in BH and 6.66 t/ha and 6.93 t/ha, in MH, respectively when compared with *R. solanacearum*-treated plots.

Table 1 Influence of *T. asperellum* on tomato plant growth, yield, and control of bacterial wilt under field conditions during the year 2014

Treatments	Plant height (cm)		Fresh weight (g)		Dry weight (g)		Stem growth (cm)		No. of fruits/plant		Yield (t/ha)		Disease incidence (%)	
Plots	BH	MH	BH	MH	BH	MH	BH	MH	BH	MH	BH	MH	BH	MH
Control	58.23^{d}	57.17^{cd}	417.32^{h}	415.76^{h}	46.32^{c}	43.45^{c}	1.18^{e}	1.09^{d}	43.25^{h}	42.67^{h}	7.52^{c}	7.12^{c}	0^{a}	0^{a}
RS1	36.45^{b}	35.65^{b}	168.21^{e}	160.98^{e}	15.14^{a}	14.54^{a}	0.54^{d}	0.52^{d}	13.59^{e}	13.43^{e}	1.44^{a}	1.32^{a}	91.07^{g}	89.65^{g}
RS2	35.26^{ab}	33.79^{a}	157.87^{abc}	156.67^{ab}	14.96^{a}	14.56^{a}	0.47^{ab}	0.51^{b}	15.15^{f}	14.23^{f}	1.65^{a}	1.45^{a}	89.18^{f}	88.43^{f}
RS3	36.44^{ab}	34.56^{ab}	164.47^{d}	159.34^{cd}	14.89^{a}	13.23^{a}	0.45^{a}	0.56^{ab}	13.89^{e}	12.76^{e}	1.54^{a}	1.55^{a}	88.54^{ef}	86.87^{ef}
RS4	38.24^{ab}	32.54^{a}	156.76^{abc}	154.55^{b}	15.92^{a}	15.76^{a}	0.52^{abc}	0.54^{bc}	12.22^{de}	11.23^{de}	1.62^{a}	1.23^{a}	88.68^{ef}	87.23^{ef}
RS5	34.07^{a}	35.23^{bc}	160.86^{cd}	153.76^{ab}	15.8^{a}	14.55^{a}	0.36^{d}	0.48^{de}	9.89^{ab}	8.98^{ab}	1.29^{a}	1.16^{a}	88.29^{fg}	84.65^{ef}
RS6	34.92^{ab}	36.76^{c}	155.32^{ab}	156.87^{ab}	16.75^{a}	15.78^{a}	0.48^{abc}	0.57^{d}	12.77^{de}	10.34^{e}	1.54^{a}	1.43^{a}	87.12^{e}	87.87^{f}
RS7	35.30^{ab}	34.57^{b}	158.54^{bc}	157.54^{bc}	16.30^{a}	15.83^{a}	0.50^{bc}	0.56^{ab}	8.55^{a}	13.76^{ef}	1.46^{a}	1.29^{a}	88.26^{ef}	85.45^{d}
RS8	33.89^{a}	35.43^{b}	156.46^{ab}	159.48^{c}	16.54^{a}	16.38^{a}	0.49^{abc}	0.54^{bc}	11.60^{cd}	12.48^{e}	1.61^{a}	1.57^{a}	85.59^{d}	87.78^{f}
RS9	36.37^{b}	37.87^{c}	153.23^{a}	152.93^{ab}	15.45^{a}	14.72^{a}	0.54^{bcd}	0.59^{d}	10.78^{bc}	12.12^{e}	1.69^{a}	1.54^{a}	89.60^{f}	84.54^{ef}
RS10	34.58^{ab}	36.62^{b}	157.11^{abc}	156.78^{ab}	15.48^{a}	16.76^{a}	0.55^{cd}	0.60^{cd}	12.27^{de}	11.16^{de}	1.54^{a}	1.72^{a}	89.18^{f}	89.54^{g}
T4	74.10^{e}	72.94^{e}	647.95^{i}	641.96^{h}	49.29^{d}	48.92^{d}	1.38^{f}	1.35^{f}	52.53^{i}	50.43^{i}	8.15^{c}	7.89^{cd}	0^{a}	0^{a}
T8	74.17^{e}	72.52^{e}	685.07^{j}	678.48^{j}	50.21^{d}	48.81^{d}	1.43^{f}	1.41^{f}	55.29^{j}	54.36^{j}	8.26^{c}	8.16^{d}	0^{a}	0^{a}
RS + T4	48.76^{c}	46.98^{d}	386.65^{f}	379.54^{f}	34.63^{b}	35.34^{bc}	1.13^{e}	1.12^{e}	38.26^{g}	37.65^{g}	6.74^{b}	6.63^{b}	37.27^{c}	39.89^{c}
RS + T8	49.54^{c}	46.56^{c}	392.14^{g}	385.76^{g}	35.26^{b}	34.98^{b}	1.15^{e}	1.14^{e}	39.18^{g}	38.33^{g}	6.79^{b}	6.49^{b}	35.58^{b}	40.27^{d}

Means of three replications, followed by the letters significantly different according to Duncan's multiple range tests (DMRT). Values with different alphabetical (a–j) superscripts in a column significantly different ($P \leq 0.05$)

RS Ralstonia solanacearum, *T4 and T8 T. asperellum* isolates, *BH* Bhoomishettihalli, *MH* Madanahalli

Table 2 Influence of *T. asperellum* on tomato plant growth, yield, and control of bacterial wilt under field conditions during the year 2015

Treatments	Plant height (cm)		Fresh weight (g)		Dry weight (g)		Stem growth (cm)		No. of fruits/plant		Yield (t/ha)		Disease incidence (%)	
Plots	BH	MH	BH	MH	BH	MH	BH	MH	BH	MH	BH	MH	BH	MH
Control	57.67^{d}	57.78^{d}	414.67^{h}	409.57^{i}	47.76^{cd}	45.76^{cd}	1.19^{e}	1.16^{d}	42.78^{h}	44.54^{g}	7.16^{c}	6.87^{c}	0^{a}	0^{a}
RS1	35.56^{b}	34.65^{ab}	160.56^{e}	161.44^{ef}	16.67^{a}	13.43^{a}	0.59^{d}	0.43^{cd}	14.45^{e}	13.76^{e}	1.56^{a}	1.32^{a}	87.34^{g}	89.76^{f}
RS2	34.45^{ab}	35.45^{ab}	158.67^{abc}	156.38^{cd}	15.54^{a}	15.87^{a}	0.48^{ab}	0.54^{ab}	15.45^{f}	14.45^{f}	1.49^{a}	1.45^{a}	88.34^{f}	90.43^{g}
RS3	35.58^{ab}	33.36^{ab}	163.43^{de}	160.12^{d}	15.34^{a}	13.56^{a}	0.46^{a}	0.56^{abc}	14.21^{e}	13.43^{e}	1.66^{a}	1.32^{a}	87.67^{ef}	87.56^{f}
RS4	37.43^{ab}	36.87^{ab}	159.98^{abc}	157.87^{bc}	16.92^{a}	15.66^{a}	0.54^{abc}	0.43^{a}	13.34^{de}	12.87^{ab}	1.57^{a}	1.55^{a}	87.45^{ef}	85.44^{d}
RS5	33.57^{a}	32.69^{a}	150.86^{cd}	148.55^{d}	15.25^{a}	13.43^{a}	0.46^{d}	0.41^{abc}	9.65^{ab}	12.34^{e}	1.38^{a}	1.57^{a}	89.48^{fg}	87.57^{gh}
RS6	33.76^{b}	35.54^{b}	154.78^{ab}	154.66^{d}	15.56^{a}	16.33^{a}	0.48^{bc}	0.43^{a}	14.34^{de}	14.67^{de}	1.47^{a}	1.43^{a}	86.87^{e}	86.33^{g}
RS7	34.54^{ab}	37.12^{ab}	156.45^{bc}	159.34^{cd}	15.87^{a}	13.58^{a}	0.53^{bc}	0.47^{bc}	9.98^{a}	17.98^{f}	1.47^{a}	1.76^{a}	88.66^{f}	89.66^{g}
RS8	34.87^{a}	34.65^{ab}	155.87^{ab}	155.65^{ab}	15.68^{a}	14.65^{a}	0.51^{abc}	0.56^{abc}	12.64^{cd}	12.54^{abc}	1.57^{a}	1.69^{a}	87.56^{ef}	85.34^{d}
RS9	35.56^{b}	35.34^{b}	155.57^{b}	157.23^{bc}	14.89^{a}	13.82^{a}	0.58^{bcd}	0.45^{b}	11.23^{bc}	13.23^{cd}	1.62^{a}	1.54^{a}	86.43^{f}	87.87^{e}
RS10	33.66^{ab}	32.33^{ab}	158.32^{abc}	155.89^{b}	15.98^{a}	15.69^{a}	0.59^{cd}	0.54^{bc}	12.78^{de}	14.33^{e}	1.57^{a}	1.65^{a}	88.89^{f}	88.56^{ef}
T4	75.23^{e}	69.89^{ef}	651.23^{i}	632.66^{ij}	46.56^{d}	47.89^{cd}	1.40^{f}	1.27^{f}	53.98^{ij}	50.57^{i}	7.39^{c}	7.89^{c}	0^{a}	0^{a}
T8	76.08^{e}	68.76^{f}	665.54^{j}	672.27^{j}	51.11^{d}	49.43^{d}	1.41^{f}	1.41^{h}	54.34^{j}	49.33^{g}	7.53^{c}	8.12^{d}	0^{a}	0^{a}
RS + T4	47.87^{c}	46.88^{c}	389.76^{f}	378.78^{g}	35.49^{b}	31.27^{bc}	1.17^{e}	1.14^{h}	39.12^{g}	36.68^{h}	6.67^{b}	7.14^{cd}	37.27^{c}	39.71^{d}
RS + T8	49.89^{cd}	45.76^{cd}	395.57^{g}	381.91^{h}	36.87^{b}	32.62^{c}	1.19^{e}	1.12^{e}	38.98^{g}	38.43^{gh}	6.89^{b}	6.98^{bc}	35.58^{b}	38.67^{c}

Means of three replications, followed by the letters significantly different according to Duncan's multiple range tests (DMRT). Values with different alphabetical (a–j) superscripts in a column significantly different ($P \leq 0.05$)

RS Ralstonia solanacearum, *T4 and T8 T. asperellum* isolates, *BH* Bhoomishettihalli, *MH* Madanahalli

The treatment of *T. asperellum* (T4 and T8) on the pathogen-infected plants showed significant increase in overall plant growth, including plant height, fresh weight, dry weight stem growth, and fruits per plant (Tables 1 and 2). The treatment of *T. asperellum* T4 increased plant height, fresh weight, dry weight, stem growth, and fruits per plant by 14.69 cm, 225.79 g, 18.83 g, 0.77 cm, and 28.37 fruits/plant, respectively in BH plot as compared with the diseased control and by 11.7 cm, 225.78 g, 20.79 g, 0.64 cm, and 28.67 fruits/plant respectively in MH plot as compared with the diseased control. The treatment of *T. asperellum* T8 increased plant height, fresh weight, dry weight, stem growth, and fruits per plant by 15.47 cm, 231.28 g, 19.46 g, 0.79 cm, and 29.29 fruits/plant, respectively, in BH plot as compared with diseased control and by 11.33 cm, 232 g, 20.43 g, 0.66 cm, and 29.35 fruits/plant respectively in MH plot as compared with diseased control (Table 1). In year 2015, the disease incidence in untreated control plots was nil in both plots. In *R. solanacearum*-infected BH and MH plots, it ranged from 86.43 to 89.48% and 85.34 to 90.43%, respectively. *T. asperellum*-treated plots showed an average of 36.43 and 39.19% disease incidence in BH and MH plots, respectively. The *T. asperellum* treatment decreased the disease incidence by 50.69% (RS + T4) and 52.38% (RS + T8) in BH and 48.18% (RS + T4) and 49.22% (RS + T8) in MH plots, respectively. The tomato yield in untreated control

Fig. 1 Biocontrol of bacterial wilt of tomato under field conditions using *T. asperellum* isolates. Untreated control plants (C), plants inoculated with *R. solanacearum* alone (T1), plants inoculated with *T. asperellum* alone (T2), and plants inoculated with *T. asperellum* and challenged with *R. solanacearum* (T3)

plot of BH was 7.16 t/ha and 6.87 t/ha in MH plot. The tomato yield in *R. solanacearum*-infected BH plot was about 1.38 to 1.66 t/ha and 1.32 to 1.76 t/ha in MH plot. *T. asperellum* isolate (T4 and T8)-treated plots yielded an average of 7.39 and 7.53 t/ha in BH and of 7.89 and 8.12 t/ha of tomatoes in MH, respectively. Thus, tomato yield increased by an average of 3.22 and 5.16% in BH and 14.85 and 18.20% in MH at T4 and T8 treatments, respectively as compared with the untreated control plots (Fig. 1). Plots challenge inoculated with *R. solanacearum* and treated with T4 and T8 yielded an average of 6.67 and 6.89 t/ha in BH and an average of 7.14 and 6.98 t/ha in MH plots, respectively. *T. asperellum* (T4 and T8) treatment enhanced the tomato yield by 5.29 t/ha and 5.51 t/ha in BH and 5.82 t/ha and 5.66 t/ha in MH, respectively when compared with *R. solanacearum*-treated plots (Tables 1 and 2).

The treatment of *T. asperellum* T4 increased plant height, fresh weight, dry weight, stem growth, and fruits per plant by 14.3 cm, 238.9 g, 20.34 g, 0.71 cm, and 29.47 fruits/plant in BH plot as compared with diseased control and by 14.19 cm, 230.24 g, 17.84 g, 0.73 cm, and 24.34 fruits/plant in MH plot, respectively. The treatment of *T. asperellum* T8 increased plant height, fresh weight, dry weight, stem growth, and fruits per plant by 16.32 cm, 244.71 g, 21.62 g, 0.73 cm, and 29.33 fruits/plant in BH plot and by 13.07 cm, 233.36 g, 19.19 g, 0.71 cm, and 26.09 fruits/plant in MH plot, respectively, as compared to diseased control (Table 2).

Obtained results showed the induction of plant growth, increased tomato yield and reduced wilt incidence under field conditions upon soil treatment with *T. asperellum*. This outcome supports the report of Watanabe et al. (2007) who reported the management of disease by *T. asperellum*. The root colonization is a successful major requirement for the useful effects of *Trichoderma* spp. on plants not only concerning antagonistic behavior and increase in plant growth but also for inducing systemic resistance (Rubio et al. 2014). *Trichoderma* spp. have been previously demonstrated as efficient *T. asperellum* for the control of *M. phaseolina* in melon, corn, eggplant, sorghum, and chickpea (Manjunatha et al. 2013) and for the control of *F. solani* in beans, chili, and peanuts (Qualhato et al. 2013). Different studies on applications of *Trichoderma* spp. in farming practices as biological control agents, biofertilizers, and soil amendments for the control of plant pathogens and crop development in several crop plants have been well established. *Trichoderma* is accomplished of colonizing farmyard manure, and therefore, application of colonized FYM to the soil is more suitable and helpful. This is the mainly successful method of application of *Trichoderma*, particularly for the control of soilborne diseases (Hamed et al. 2015).

Biochemical analysis

The present results showed that the isolates of *T. asperellum* significantly induced maximum levels of defense enzyme activities in tomato leaves challenged with pathogen, compared with uninoculated tomato plants. Therefore, the present study revealed that *T. asperellum* exhibited significantly induced POX, PPO, PAL, β-1,3-glucanase, and total phenolic contents in plants challenged with *R. solanacearum* in tomato plants (Figs. 2, 3, 4, 5, and 6). The native gel electrophoresis also indicated the induction of isoforms in POX and PPO. Increase in activity and accumulation of systemic resistance enzymes also depends on the plant genotype, physiological conditions and the type of pathogen. Synthesis of defense chemicals against pathogens is triggered by a series of morphological and biochemical changes initiated by specific strains of fungi (Siva Prasad et al. 2013). Therefore, treatment of tomato seedlings with *T. asperellum* isolates induced POX, PAL PPO, β-1,3-glucanase, and total phenolic contents in plants infected with *R. solanacearum*. At 12 days after inoculation, the significantly increased POX activity was observed in treatment with *T. asperellum* isolates upon challenge inoculation with *R. solanacearum* and declined thereafter in all the treatments. Plants treated with *T. asperellum* alone also showed enhanced activity as compared with *R. solanacearum* treated and untreated control (Fig. 2). POX is a component of an early response in plants to pathogen infection and plays a key role in the biosynthesis of lignin, which limits the extent of pathogen spread (Vidhyasekaran 2008). When POX level increases due to the induced systemic resistance, a quick synthesis of reactive oxygen derivatives by oxidative burst leads to cell death and inhibits pathogenic activities that were observed (Prasannath et al. 2014). Therefore, improvement of POX level on 12th day after pathogen inoculation in tomato plant leaves is considered to be the marker for the development of disease resistance against *R. solanacearum*. Obtained results were supported by earlier studies which demarcated the induction of POX in plants infected by pathogens, resulting in faster and stronger resistance against them (Surekha et al. 2014). They experimentally supported the idea that peroxidase plays a defense role against attacking pathogens (Caruso et al. 2001).

PPO activity in tomato plants treated with *T. asperellum* was significantly increased upon challenged with pathogen and reached at 14th day and declined thereafter in all the treatments (Fig. 3). It is copper containing enzymes that catalyze oxidation of hydroxy phenols to their quinone derivatives, which have antimicrobial activity (Chunhua et al. 2001). Oxidative enzymes such as POX and PPO can catalyze the formation of lignin and other oxidative phenols and contribute in the formation

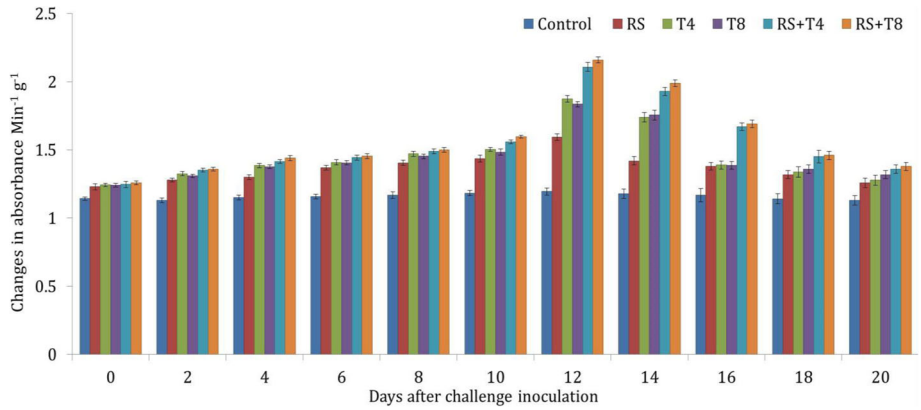

Fig. 2 Changes in peroxidase (POX) activity in tomato plants of different treatments. Mean values were three replicates. Bars represent standard error. Plants inoculated with *R. solanacearum* alone (RS); plants inoculated with *T. asperellum* alone (T4 and T8); *T. asperellum*-treated tomato plants challenged with *R. solanacearum* (RS + T4 and RS + T8)

of defense barriers by changing the cell structure defense system that gets actuated against pathogens (Li and Steffens 2002). Several potentials of PPO including general toxicity of PPO-generated quinones to pathogens and plant cells, accelerating cell death, alkylation, and reduced bioavailability of cellular proteins to the pathogen, crosslinking of quinones with protein or other phenolics, forming a physical barrier to pathogens in the cell wall and quinone redox cycling leading to H_2O_2 and other reactive oxygen species. In the present experiment, PPO activity was significantly enhanced by *T. asperellum*-treated tomato plants. Also, PPO activity level increased at 14th day after challenge inoculation and helps in disease resistance as it oxidizes the phenolic level increase during this stage to toxic molecules such as quinones leads to invasion of pathogen (Vinale et al. 2008). Activity of PAL in tomato plants treated with *T. asperellum* was significantly increased in tomato plants inoculated with *R. solanacearum*. The PAL activity reached maximum at 10th day after challenge inoculation with the pathogen and declined thereafter in all the treatments. Activity of PAL in tomato plants treated with *T. asperellum* was induced upon inoculation with pathogen (Fig. 4). Induction of defense enzymes like PAL is one of the responses of the host for treatment with *Trichoderma* agents. PAL is the key enzyme that is responsible for linking primary metabolism of aromatic amino acids with secondary metabolic products (Macdonald and Dcunha 2007). It is the first enzyme in phenyl propanoid metabolism and synthesis of various phenolic compounds as well as anthocyanin, biosynthesis of lignin providing mechanical strength to the plant cell wall and phytoalexins which are responsible for prevention of establishment of plant pathogens (Karthikeyan et al. 2005). In the present study, increased PAL activity and the accumulation of phenolic content

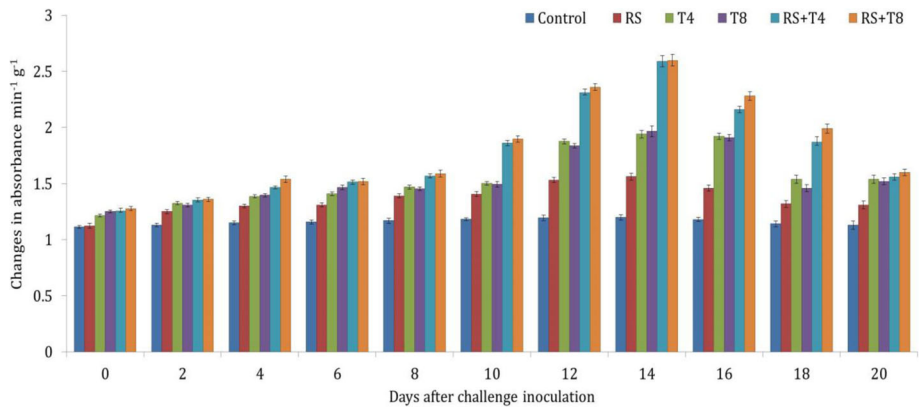

Fig. 3 Changes in polyphenol oxidase (PPO) activity in tomato plants of different treatments. Mean values were three replicates. Bars represent standard error. Plants inoculated with *R. solanacearum* alone (RS); plants inoculated with *T. asperellum* alone (T4 and T8); *T. asperellum*-treated tomato plants challenged with *R. solanacearum* (RS + T4 and RS + T8)

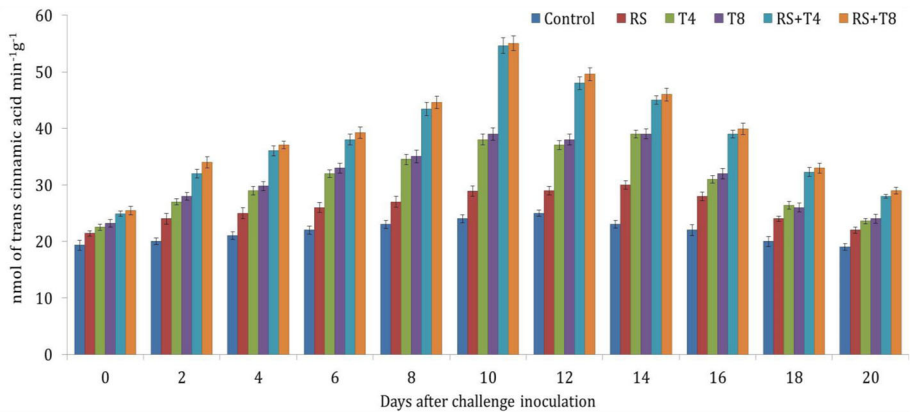

Fig. 4 Changes in phenylalanine ammonia lyase (PAL) activity in tomato plants of different treatments. Mean values were three replicates. Bars represent standard error. Plants inoculated with *R. solanacearum* alone (RS); plants inoculated with *T. asperellum* alone (T4 and T8); *T. asperellum*-treated tomato plants challenged with *R. solanacearum* (RS + T4 and RS + T8)

was recorded in *T. asperellum* isolates treated tomato plants infected with the *R. solanacearum*, apparently due to prevention of pathogen attack. Also, the *T. asperellum* treatment resulted in a significant increase in the PAL activity on 10th day after pathogen inoculation in tomato plants.

The β-1,3-glucanase activity reached the maximum at 12th days after inoculation with *R. solanacearum* and declined thereafter in all the treatments (Fig. 5). Improved level of pathogenesis-related PR protein such as β-1,3-glucanase activity was observed in *T. asperellum*--treated tomato plants and leads to disease resistance against *R. solanacearum*. It is a member of the PR protein family, known to directly destroy pathogen cell walls. Improved β-1,3-glucanase activity was observed by *T. asperellum* level up to the 12th day after pathogen inoculation, and thereafter, it starts decreasing leading to disease resistance in tomato plants against *R. solanacearum*. Similar outcomes were previously approved by Saksirirat et al. (2009) who demonstrated that the increase in PR proteins, like chitinase and β-1,3-glucanase level up to 14th day of *X. campestris* pv. *vesicatoria* inoculation on tomato plants, led to the leaf spot.

Accumulation of phenolics in plants pre-treated with *T. asperellum* was induced upon challenge inoculation with *R. solanacearum*. Its accumulation significantly increased on 10th days after inoculation with the pathogen and declined thereafter in all the treatments. Maximum accumulation of phenol was noticed in *T. asperellum* (T8) inoculated with *R. solanacearum* at 10 days when compared with the plants inoculated with the pathogen alone (Fig. 6). Minimum amounts of phenolic compounds were observed in untreated control. The development of production of phenolics, known as defense molecules of plants against plant pathogens and insects, is indicated by an increase in PAL activity in wounded

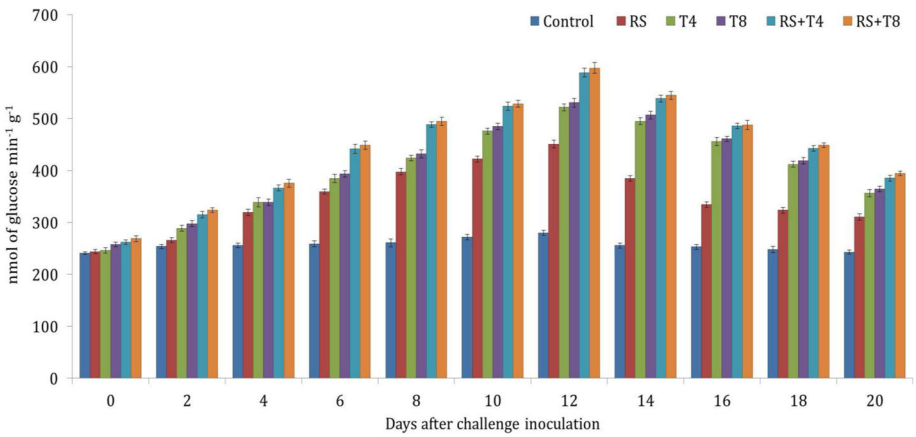

Fig. 5 Changes in β-1,3-glucanase activity in tomato plants of different treatments. Mean values were three replicates. Bars represent standard error. Plants inoculated with *R. solanacearum* alone (RS); plants inoculated with *T. asperellum* alone (T4 and T8); *T. asperellum*-treated tomato plants challenged with *R. solanacearum* (RS + T4 and RS + T8)

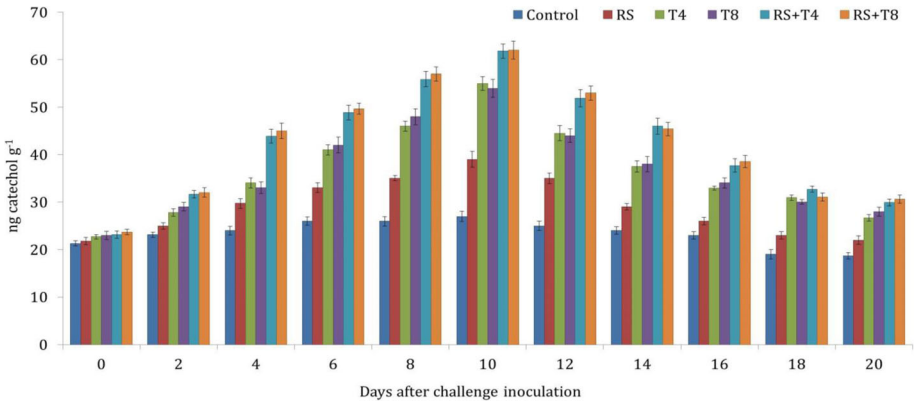

Fig. 6 Changes in total phenols in tomato plants of different treatments. Mean values were three replicates. Bars represent standard error. Plants inoculated with *R. solanacearum* alone (RS); plants inoculated with *T. asperellum* alone (T4 and T8); *T. asperellum*-treated tomato plants challenged with *R. solanacearum* (RS + T4 and RS + T8)

plant tissues (Bi and Felton 1995). In the present study, a high quantity of phenolic compounds was observed in the plant as compared with treated and untreated controls when tomato seedlings were treated with *T. asperellum*. Investigation data accumulated in the past few years have produced a completely novel understanding of the way by which these fungi interact with plants. Lopes et al. (2012) described a positive correlation between the lytic enzyme activities and the antagonism capacity of *T. asperellum* against *S. sclerotiorum*. The presence of *T. asperellum* in cucumber roots triggers the SA and JA pathways in the plant and increased peroxidase activity, hence conferring protection to cucumber plants against foliar pathogens (Segarra et al. 2007).

Native PAGE analysis POX and PPO

Native PAGE analysis revealed that POX isoforms designated as POX1 to POX8 were observed in *T. asperellum*-treated tomato leaf tissues inoculated with the *R. solanacearum* and the expression of isoforms POX3, POX4, POX6, and POX7 showed higher induction in tomato plants inoculated with the pathogen compared with other treatments under field conditions (Fig. 7a). Similarly, five isoforms of PPO (PPO1 to PPO5) were showed in *T. asperellum*-treated plants after inoculation with *R. solanacearum*, whereas in the controls less intensity was noticed. A higher induction of PPO was observed in plants pretreated with *T. asperellum* inoculated with *R. solanacearum* (Fig. 7b). The increased

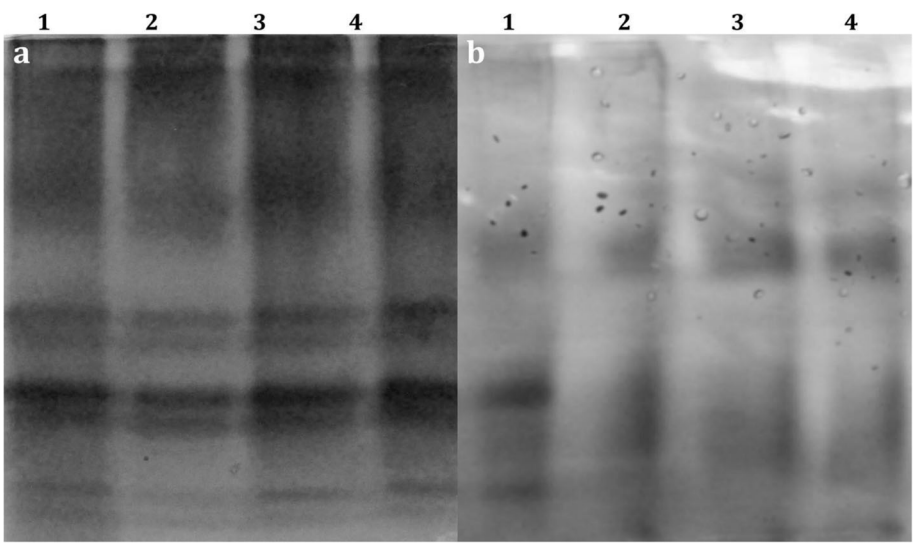

Fig. 7 Native PAGE profiles of (**a**) POX and (**b**) PPO, induced in response to challenge with *R. solanacearum*. Lane 1—untreated control (C), lane 2—*R. solanacearum* alone (T1), lane 3—*T. asperellum* alone (T2), and lane 4—inoculated with *T. asperellum* and challenged with *R. solanacearum* (T3)

expression of isoforms of POX and PPO might be responsible for reduced disease incidence.

Conclusions

The present study showed that biocontrol capacity and biochemical characterization of induced systemic resistance by *T. asperellum* against *R. solanacearum* in tomato and comprehends the role of defense enzymes in developing disease resistance under field conditions. The two *T. asperellum* isolates recorded efficient inhibition against *R. solanacearum* and increased yield of tomatoes under field experiments. The role of *T. asperellum* is as BCA in the induction of a series of defense responses such as accumulation of phenols and induction of POX, PPO, and PAL enzymes involved in phenylpropanoid metabolism and of PR protein (β-1,3-glucanase) in response to treatment with the biocontrol agent. In this regard, it is recommended the use of *T. asperellum* assessment to the actions within disease management framework is reasonable, provided that long term induced resistance and should be developed as a sustainable and environmental friendly approach.

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Availability of data and materials

All datasets on which conclusions of the study have been drawn are presented in the main manuscript.

Authors' contributions

NK conceived the idea, suggested the point of research, designed the experimental work, conducted the experiments, and wrote the manuscript. SK worked on the management of the article, statistical analysis of data, and critical revision. SCN, SRN, and SC participated in the experiments' design and coordination. All authors read and approved the final manuscript.

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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