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A synergic and compatible microbial-based consortium for biocontrol of Fusarium wilt of tomato

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Summary. Bioconsortia, based on *Chaetomium globosum* (isolate CgCG-2), *Pseudomonas putida* (PpTS-1), *Bacillus subtilis* (BsS2BC-1), and *Trichoderma harzianum* (ThS17TH), were designed to develop eco-friendly alternatives for biocontrol of vascular wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*). *In vitro* compatibilities of microbes in these consortia were assessed for growth, antagonism, and biocontrol-related gene expression. In these bioassays, the biocontrol isolates had positive interactions for the tested parameters. In pot experiments, seed and soil applications of culture suspensions of five different isolate mixtures were assessed, in comparisons with individual isolates, for efficacy in vascular wilt control after challenge inoculations with *Fol* under polyhouse conditions. Compared to experimental controls, the biocontrol isolate mixtures reduced vascular wilt incidence and promoted plant growth. PpTS-1 + CgCG-2 + ThS17TH was the most effective microbial consortium, giving 71% reduction of Fusarium wilt incidence compared to non-treated controls. This reduced incidence increased plant growth by 135%. Upregulation of genes encoding for allene oxide cyclase, pathogenesis-related proteins 3, and 5, and β-1,3-glucanase in tomato plants indicated that the reduction in vascular wilt by the consortia could be partly plant-mediated. This study provides new insights into the development of microbial-based consortia for the biocontrol of vascular wilt in tomato.

Keywords. *Chaetomium*, bioconsortium, tomato, Fusarium wilt, management.

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INTRODUCTION

Vascular wilt, caused by *Fusarium oxysporum* f. sp. *lycopersici* W. C. Snyder & H. N. Hans (*Fol*), is an important disease of tomato. This disease causes yield losses (25 to 55%) and tomato fruit quality reductions (Sidharthan *et al.,* 2018; Sidharthan *et al.,* 2019). This disease is currently being managed with fungicides, but without adequate control. The use of chemicals may affect the environment and cause development of fungicide-resistant pathogen isolates, and is expensive (Shanmugam and Kanoujia, 2011). The use of

resistant cultivars for disease management may be constrained by development of new physiological races of pathogens (McGovern, 2015, Shanmugam *et al*., 2011a). Due to problems associated with the use of chemicals and resistant cultivars (Shanmugam and Kanoujia, 2011), biomanagement of diseases using plant growth-promoting rhizobacteria (PGPR's), particularly fluorescent pseudomonads and *Bacillus* spp., or *Trichoderma* spp., may be an alternative disease management strategy (Aggarwal, 2015), also because these microbes can have growth-promoting activities in crop plants (Jetiyanon and Kloepper, 2002).

In biocontrol of plant diseases, PGPRs and *Trichoderma* spp. act directly on the pathogens by competing for nutrients, space and ecological niches, or by producing antimicrobial compounds, and/or indirectly, through induction of host plant defence mechanisms (induced systemic resistance). This has been reported to be broad-spectrum and long-lasting (Shanmugam and Kanoujia, 2011). Besides these microbes, species of *Chaetomium* (*Ascomycetes*) can have potential for the biocontrol of plant diseases (Aggarwal, 2015). *Chaetomium* spp. commonly exist in soil or organic compost, and are exploited by biotechnology industries due to their ability to produce enzymes such as cellulase and laccase. Among *Chaetomium* spp., *Chaetomium globosum* has been widely used for biocontrol of soilborne and foliar plant pathogens (Aggarwal, 2015; Aggarwal *et al.,* 2016; Darshan *et al*., 2020).

Biocontrol mechanisms of *Chaetomium* spp. are generally attributed to secretion of cell wall-degrading enzymes and antibiotics. They are also known for plant growth stimulation and induction of host resistance (Aggarwal, 2015). Induced resistance by single biocontrol agents of *Bacillus* spp., fluorescent pseudomonads, or *Trichoderma* has been reported, but mechanisms for biocontrol consortia have been less publicized (Shanmugam and Kanoujia, 2011; Shanmugam *et al.,* 2013). Induction of defence enzymes has mostly been implicated in plant-mediated resistance to pathogens (Shanmugam and Kanoujia, 2011; Shanmugam *et al.,* 2013; Aggarwal, 2015).

Fluorescent pseudomonads and sporulating Gram-positive bacteria (e.g. *Bacillus* spp.) have been extensively used to manage a broad spectrum of plant pathogens, including *Fusarium* in many plant species (van Loon *et al.,* 1998; Kloepper *et al.,* 2004). Likewise, *Trichoderma* spp. have also been commonly reported as potential biocontrol agents against *Fusarium* and other pathogens that incited diseases in several crop plants (Asad, 2022). In tomato, fluorescent pseudomonads and species of *Bacillus* or *Trichoderma* have been reported to manage Fusarium wilt (Shanmugam *et al.,* 2011b; 2015). In the biomanagment of crop diseases, deploying a single antagonist often results in inconsistent field performance, due to poor stability of the antagonist in different soil environments (Shanmugam and Kanoujia, 2011). Improved efficacy of biocontrol consortia compared with individual antagonists, has been reported, by employing combinations of bioagents (Shanmugam *et al.,* 2013). These bioagents exhibit different modes of action, and occupy different or complementary niches (Larkin and Fravel, 1998).

Colletotrichum globosum could be a candidate in a biocontrol consortium, as this fungus can produce several secondary metabolites. However, inappropriate use of antagonists in combinations may reduce their biocontrol efficacy (Shanmugam *et al.,* 2002; Shanmugam and Kanoujia, 2011; Shanmugam *et al.,* 2013). Therefore, antagonists designated for isolate mixtures must be carefully evaluated to exploit their fullest potential (Shanmugam *et al.,* 2011b; Shanmugam *et al.,* 2013). Management of Fusarium wilt of tomato involving PGPRs (Shanmugam and Kanoujia, 2011; Shanmugam *et al.,* 2011b), or PGPRs and *Trichoderma* spp. (Shanmugam *et al.,* 2013), has been reported. *C. globosum,* despite being a potential biocontrol agent, has seldom been exploited for *Fol* management in tomato either individually or as a consortium.

The present study aimed to test the antagonistic activities of *Bacillus subtilis*, *Pseudomonas putida*, *Trichoderma harzianum* and *C. globosum* against *Fol*, and design a synergic and compatible microbial consortium exhibiting *in vitro* compatibilities for host plant growth promotion, antagonism and biocontrol gene expression, as attributes of a biocontrol agent for management of vascular wilt control in tomato. Expression of candidate defence-related genes in tomato were also profiled, to identify possible plant-mediated mechanisms of the effective *Chaetomium*-based biocontrol.

MATERIALS AND METHODS

Fungal pathogen and biocontrol isolates

A highly virulent *Fol* isolate, TOFOL-IHBT (Sidharthan *et al.,* 2018) was used as the pathogen. To prepare inoculum, the fungus was cultured on Potato Dextrose Agar (PDA) in Petri plates, for 7 d at 28°C. Conidium suspension of the isolate were prepared by pouring 20 mL of sterile distilled water into each Petri plate. The suspension was filtered through fine nylon mesh to remove large mycelial parts, was quantified using a haemocytometer, and then adjusted to 10^4 conidia mL^{-1}.

These suspensions were used as inoculum after mixing with 0.05% Tween 80. Either mycelium maintained on the PDA or conidium suspension was used as the pathogen inoculum.

Potential rhizobacterial isolates, TEPF-Sungal-1 (PpTS-1, *P. putida*, GenBank No. MZ363827.1) and S2BC-1 (BsS2BC-1, *B. subtilis*, GenBank No. AM268039), and a *T. harzianum* isolate, S17TH (ThS17TH, GenBank No. GU048855) and a *C. globosum* isolate, CG-2 (CgCG-2, GenBank No. AY429049), elsewhere reported as effective biocontrol agents against soilborne and foliar diseases of ginger and wheat (Shanmugam *et al.,* 2013; Aggarwal *et al.,* 2016), were obtained from laboratory collections, and were assessed as biocontrol isolates for control of vascular wilt of tomato. Pure cultures of the rhizobacterial and fungal isolates were obtained by sub-culturing on nutrient agar/King's B agar for the rhizobacteria, or potato dextrose agar (PDA) for the fungi, and purified cultures were maintained at 28 ± 2°C.

Evaluation of isolates for antagonism

The isolates were tested *in vitro* for antagonism against the pathogen by dual culture (DC) assays on PDA plates, in triplicate. In each assay, inoculation of the antagonistic isolate was timed with respect to the growth rate of the pathogen. A 5 mm mycelial disc taken from 7-d-old *Fol* culture was inoculated to the near periphery of one half of a Petri dish containing PDA. A loopful of a 48 h bacterial culture was streaked perpendicular to the pathogen on the opposite side towards the edges of the plate. A mycelial disc (5 mm diam.) of fungal antagonist was placed at the edge of the opposite half of the Petri dish. PDA plates inoculated with the pathogen alone served as experimental controls. Antagonistic effects of the bacterial isolates and CgCG-2 were recorded by measuring the inhibition zones from the bacterial streaks or the edges of the antagonist mycelium to the edges of the pathogen mycelium. Growth inhibition by the *T. harzainum* isolate was assessed using the scale of Shanmugam *et al.* (2013).

The biocontrol isolates were also assessed for antagonism using a 10% cell-free culture filtrate (CFC) using the technique of Vaidya *et al.* (2004). To obtain the CFC filtrates, 50 mL of sterile Potato Dextrose Broth (PDB) in a 250 mL capacity conical flask was inoculated separately with a loopful of *Pseudomonas* grown on nutrient agar or *Bacillus* colonies from King's B agar medium. For *Trichoderma* and *Chaetomium*, 5 mm mycelial discs of actively growing young cultures grown on PDA were used. The flasks were then incubated in shake cultures, at 28 ± 2°C for 2 d for the bacteria, 7 d for *Trichoderma*, or 10 d for *Chaetomium*. These cultures were then coarse-filtered using Buckner flasks. Each filtrate was again filtered through a Millipore filter (pore size 450 nm), and the required quantity of the filtrate was aseptically mixed with a measured volume of PDA slurry to obtain the desired (10%) concentration. The medium was poured into sterile Petri plates (90 mm diam.) and solidified. Mycelial discs (5 mm diam.) were taken from a 7-d-old culture of *Fol*, and placed at the centre of each Petri plate. Plates with only the PDA medium (without the filtrate) were also inoculated with the pathogen as experimental controls. The plates were incubated at 28 ± 2°C, and mycelium growth was measured diametrically when growth in the control plates reached reached the plate perimeters. Mycelial growth inhibition was calculated using the following formula: inhibition of growth (%) = (C–T/C) × 100, where C is the average mycelial growth (diameter in mm) for three replicates in the control, and T is the average for three replicates of growth (diameter in mm) for the treatment plates.

Antifungal effects of extracellular metabolites of biocontrol isolates

Mutual inhibition among the biocontrol isolates was initially assessed by the DC and CFC filtrate assays in triplicate, as described earlier, to elucidate their compatibility. Later, each of the biocontrol isolates was evaluated in triplicate for their antagonistic efficacies against the fungal pathogen by the DC assay on PDA in the presence of CFC (10% v/v) of the other biocontrol isolates prepared as described earlier. PDA alone without CFC filtrate served as a control.

Inhibitory effects of extracellular metabolites of biocontrol isolates

Compatibility among the biocontrol isolates was also assessed by testing CFC filtrates (10% v/v) of each isolate for their inhibitory effects on growth of the other isolates. For effects of isolates ThS17TH and CgCG-2 on the growth of rhizobacteria, the CFC filtrates of the fungi were prepared as described above, and were incorporated separately into nutrient broth in triplicate. The broth was then inoculated with bacterial cell suspensions grown for 24 h (1.5×10^8 cfu mL^{-1}). Population of the rhizobacteria was then assessed after 48 h incubation in a rotary shaker, by observing the optical density of the broth in a colorimeter at 600 nm. The cultures of the rhizobacteria without the filtrate were used as experi-

mental controls. Either an increase or no change in the population densities of the treatments compared with the control was considered a positive interaction.

To test the effects of isolates PpTS-1 and BsS2BC-1 on the antagonistic fungi, their respective CFC filtrates, prepared as described above, were incorporated into PD broth (at 10% v/v), and the broth was inoculated with the respective fungal spore suspensions (1×10^5 conidia mL^{-1}). After incubation at 28 ± 2°C for 7 d on a rotary shaker (180 rpm) in the dark, the resulting mycelium was filtered through pre-weighed filter paper, and the mycelium weight was recorded after drying at 65°C for 4 h. PDB alone was used as the experimental control.

Effects of differentially treated rhizobacteria and Trichoderma *isolates on mycelial growth and lytic enzyme production by isolate CgCG-2*

Cells of each isolate were differentially treated, and the differentially treated bacterial cells or spores of isolate ThS17TH were evaluated in triplicate for effects on the growth of isolate CgCG-2. An inductive fermentation broth consisting of cell suspensions (grown for 24 h) of either isolate BsS2BC-1 or PpTS-1 was amended with 1.5×10^8 cells mL^{-1} into 100 mL of minimal synthetic broth (MSB) (0.2 g of $MgSO_4.7\ H_20$, 0.9 g of K_2HPO_4, 0.2 g of KCl, 1.0 g of NH_4NO_3, 2 mg of $FeSO_4.7\ H_20$, 2 mg of $ZnSO_4.7\ H_20$, 2 mg of $MnCl_2.7\ H_20$, supplemented with 0.1% glucose; pH 5.6), and was then incubated on a rotary shaker (200 rpm) for 48 h. For live cells, optical density of the broth was adjusted to 0.7 at 600 nm (1.5×10^8 cells mL^{-1}) with sterile distilled water. For isolate ThS17TH, a conidium spore suspension was prepared as described above for *Fol*, and was amended to 100 mL with MSB. The cell/conidium suspensions were then each inoculated with 1 mL of conidial suspension (1×10^5 conidia mL^{-1}) prepared from a 5-d-old isolate CgCG-2 culture, and then incubated at 28±2°C for 5 d in shake cultures. The resulting mycelium was collected by centrifugation (5000 rpm, 15 min) and was weighed on sterile Whatman No. 1 filter paper. The weighed mycelia were fine-ground with liquid nitrogen, and were then used for biocontrol gene expression analyses. For heat-inactivated cells, the bacteria-amended MSB was sterilized, and the mycelium was collected as described above. Non-inductive ferment broths without the bacterial suspensions/ThS17TH conidia were similarly prepared as experimental controls.

The CFC filtrates of isolate CgCG-2 grown in MSB alone or MSB amended with heat-inactivated or live cells/conidia were also obtained, and were used as enzyme sources for estimating extracellular enzyme activities. Activity of chitinase (*N*-acetyl-β-D-glucosaminidase, NAGase) of the crude extracellular filtrate was assessed using a chitinase assay kit (Sigma), following the manufacturer's protocol. One unit of chitinase was defined as the amount of the enzyme that released 1.0 µmole min^{-1} of *p*-nitrophenol from 4-nitrophenyl- *N*-acetyl-b-D-glucosaminide, at pH 4.8 and 37°C. For β-1,3-glucanase, the reaction mixture contained 500 µL of 5.0% (w/v) laminarin (Hi-media) in 50 mM acetate buffer (pH 4.8) and 200 µL supernatant from each culture filtrate. The mixture was incubated at 45°C for 30 min, and the reducing sugar produced was determined using dinitrosalicylic acid (El-Katatny *et al.,* 2000). The amount of reducing sugars released was calculated from standard curves recorded for glucose, and enzyme activity was expressed as nkat (nmoles sec^{-1}) of glucose released per mL of culture filtrate. One unit of β-1,3-glucanase liberated 1 mg min^{-1} of reducing sugar (measured as glucose) from laminarin, at pH 5.0 and 37°C.

Effects of differentially treated potential biocontrol isolates on chitinase gene expression in isolate CgCG-2

Total RNA was isolated from 100 mg of isolate CgCG-2 mycelium using Tri-reagent (Life Tech), as per the manufacturer's instructions. The concentration and purity of RNA were quantified using a nano-Drop ND-100 (Nano-Drop Technologies). The total RNA was treated with DNase (Thermo Scientific), according to the manufacturer's guidelines. First-strand cDNA synthesis was carried out using 1 µg of total RNA and 1 µL of oligo (dT)18 (0.5 µg or 100 pmol), using a first-stand cDNA synthesis kit (Thermo Scientific), following the manufacturer's protocol. The qRT-PCR assay was conducted in triplicate along with a non-template control in a BioRad iCycler, with an initial denaturation for 180 s at 95 °C, 40 cycles of 95°C for 15s, 58°C for 20 s, and 72°C for 20 s, followed by melt curve analysis**.** Alpha-tubulin was used for the normalization of chitinase gene expression. Each 20 µL reaction mixture included 2 µL of the cDNA template, 10 µL of SYBR Green PCR Master Mix (Thermo Fisher) and 250 nM final concentrations of each forward and reverse primer specific to the chitinase gene (Table 1). Comparison of relative gene expression fold changes between the treatments was determined according to the ΔΔCt method (Livak and Schmittgen 2001).

Preparation of biocontrol inoculum for pot experiments

Bacterial inoculum was prepared by growing each bacterial isolate in an Erlenmeyer flask (250 mL capac-

Table 1. qRT-PCR primers used in this study.

Organism	Gene	Primer sequence (5'-3')	Reference
Solanum lycopersicum	Cyclopilin (*CYP*)	GAGTGGCTCAACGGAAAGCA CCAACAGCCTCTGCCTTCTTA	Shavit *et al.* (2013)
	Pathogenesis-related protein 5 (*PR 5*)	AAACGGTGAATGCCCTGGTTCA AGGACCACATGGACCGTGATTA	
	Pathogenesis-related protein 3b (*PR 3b*)	GCCCAAACTTCCCATGAAAC CAAGGCCATTGACTACTTGGTG	Sotoyama *et al.* (2015)
	β-1,3-glucanase (*glu B*)	CGAGATGGTGGGTACAGAAGAAC CAAGATTGGAAGTGCCAGTAACAGG	Martínez-Medina *et al.* (2013)
	Allene oxide cyclase (*AOC*)	GCACGAAGAAGAGAAGAAAGGAGAT CGGTGACGGCTAGGTAAGTTTC	Uppalapati *et al.* (2005)
Chaetomium globosum	Endochitinase (*chi 46*)	AGGTGCTGGCGGATTATGACG CACATTCCCAATCAGACTCTCG	Liu *et al.*, (2008)
	α-tubulin	CCTACGCGCCCGTTGTCTC GAACTGGATGGTGCGCTTGG	

ity) containing 150 mL of King's B (for isolate PpTS-1) or nutrient broth (for BsS2BC-1) for 48 h at 28 ± 2°C on a rotary shaker. The resulting cells were removed by centrifugation at 6000 g for 12 min at 4°C, and then washed in sterile distilled water. The pellet was resuspended in 0.2 M sodium phosphate buffer (pH 7.0), and the population was adjusted to 3×10^9 cfu mL^{-1} (0.5 OD at 600 nm = 1×10^8 cfu mL^{-1}). To prepare the inoculum of each fungal biocontrol isolate, a 5 mm mycelial disc was inoculated into 150 mL of PDB, and incubated for 10 d at 28 ± 2°C on a rotary shaker. The mycelium was then removed after passing the culture through muslin cloth, and the spore (or conidium) suspension was used as inoculant after adjusting the concentration to 3×10^7 cfu mL^{-1}. For bioconsortia, the biocontrol isolates designated for mixtures were grown separately on their respective media at 28 ± 2°C, and equal volumes (v/v) of the isolates were used as inoculants.

Polyhouse evaluation of selected antagonists for Fusarium wilt management

Experiments with tomato ('Pusa Rohini') were carried out over 90 d (from the day of sowing), in a polyhouse (75% RH and 18–20°C), using a completely randomized experimental design with six replicates of 12 plants per treatment. Single isolates or a designated mixture of the biocontrol isolates were applied as seed treatments or soil applications. For each seed treatment, the tomato seeds were surface sterilized with 1% sodium hypochlorite for 5 min, and were then soaked in the microbial inoculum (10^4 spores/conidia mL^{-1}). After 12 h, the suspension was drained off, and the seeds were dried for 30 min. The treated seeds were then sown (25 seeds per pot) in 30 cm diam. pots each containing 15 kg of steam-sterilized soil. After 30 d, seedlings were thinned to three seedlings per pot, and 15 d later, the remaining seedlings were inoculated with 50 mL of *Fol* conidium suspension. The microbial inoculants were also applied as soil applications, with the first application made 1 d after pathogen inoculation, and a second application at 15 d later. Non-microbe treated seeds treated with *Fol* served as pathogen experimental controls. Carbendazim (0.1%) applied as a seed treatment, or soil application at 0.2% (i.e., 2 g ai kg^{-1} seed or pot) served as fungicide controls. Vascular wilt development on each tomato plant was rated at 14 d after pathogen inoculations, and a proportional (%) disease index (PDI) was calculated, as described by Shanmugam and Kanoujia (2011). Plant heights (root + shoot lengths) was recorded at the time of harvest to assess plant growth-promoting activity of the biocontrol isolates.

Tissue collection and defence gene expression assay

To understand the role of tomato defence genes in disease suppression by the biocontrol isolates, root samples (one per replication for each treatment; six plants per treatment) were collected at 3 or 6 d post-inoculation (dpi) with the pathogen. The samples were washed in running tap water, and were each homogenized with liquid nitrogen in a pre-chilled mortar and pestle. The

homogenized root tissues were stored at −80°C. Total RNA extraction, DNase treatment, cDNA synthesis and qRT-PCR analyses were carried out, using the tomato primer pairs listed in Table 1. In the quantitative analyses, cyclophilin (*cyp*) was used as the housekeeping gene to balance the amount of cDNA in the samples.

Statistical analyses

All the experiments were replicated as described, and carried out in completely randomized designs. The pot culture experiments were repeated with similar results, so results from one representative trial are presented. Statistical analyses were carried out using the package IRRISTAT version 92-1, developed by the International Rice Research Institute Biometrics Unit, the Philippines. Differences between treatment mean values were determined following LSD tests (at $P = 0.05$).

RESULTS

Antagonism of potential biocontrol isolates against Fusarium oxysporum

In the DC assays, the isolates BsS2BC-1, PpTS-1, ThS17TH and CgCG-2 all exhibited antifungal activity against *Fol* compared to the controls. Among them, isolate BsS2BC-1 gave mean mycelium growth inhibition of 47.3%, PpTS-1 gave 38.2%, and CgCG-2 gave 41.9% inhibition, and all these isolates displayed zones of inhibition, indicating antibiosis. Isolate ThS17TH exhibited mycoparasitic activity and was classified as producing class I antagonism by overgrowing the pathogen and completely covering the medium surfaces (Table 2 and Supplementary Figure 1).

In the CFC assays, similar to the DC assays, the CFC of antagonists inhibited mycelial growth of *Fol*, and the mean mycelial growth inhibition ranged from 28.7% (isolate ThS17TH) to 59.6% (isolate PpTS-1) compared with control, (data not shown; Supplementary Figure 2). Inhibition of the pathogen by isolate ThS17TH to a level comparable with that of the other biocontrol isolates indicated that this isolate exhibited antibiosis in addition to mycoparasitism (Table 2).

Antifungal effects of extracellular metabolites from isolates

In the DC assays for mutual inhibition of the potential biocontrol isolates, no inhibition of the antagonistic fungi was displayed by either of the two rhizobacterial isolates. No mutual inhibition was observed between the two fungal isolates ThS17TH and CgCG-2. Likewise, in the antagonism assay, none of the CFC filtrates from the potential biocontrol isolates inhibited mycelial growth of the other isolates (data not shown; Supplementary Figures 3 and 4). The lack of growth inhibition in the confrontation and antagonism assays indicated compatibility among the isolates. In further studies on the antagonistic effects of each of the biocontrol isolates against the pathogen in the presence and absence of CFC filtrate of each of the other antagonists, the four isolates all exhibited similar or greater efficacies on PDA amended with the CFC filtrate in comparison to their efficacies on the medium without the filtrate. In the presence of CFC filtrate, among the biocontrol isolates, BsS2BC-1 exhibited greatest efficiency (24.5% inhibition) compared with the control, on PDA amended with the CFC filtrate of isolate PpTS-1. In contrast, the antagonistic efficiency of isolate BsS2BC-1 was less (10.9%) on PDA amended with the CFC of isolate PpTS-1. However, isolate PpTS-1 displayed slightly lesser levels of the antagonism (2.9% to 6.7%) compared with the control on PDA amended with the CFC filtrates of isolates BsS2BC-1, ThS17TH and CgCG-2. Like the rhizobacteria, the antagonistic fungal isolate CgCG-2 generally displayed less (0.6%), or better efficacies (2.5% to 7.4%) on PDA amended with the CFC filtrate of the other antagonists in comparison to their efficacies on the medium without filtrate. However, isolate ThS17TH displayed similar levels of the antagonism (class I) on PDA in the presence and absence of the CFC filtrate (Table 3; Supplementary Figure 5). These experiments demonstrated the general non-inhibitory effects of CFC of any of the antagonists on the antifungal activity of the other potential biocontrol isolates.

Extracellular metabolite effects biocontrol isolates on their growth

In the colorimetric assays, growth of both rhizobacterial isolates, BsS2BC-1 and PpTS-1, in the MSB amended with the CFC filtrate of any of the other three biocontrol isolates, was significantly greater than that of the control treatment. Among the isolates, BsS2BC-1 exhibited greater population density (5.7×10^8 cfu mL^{-1}) in the presence of CFCs of isolate PpTS-1, which was 1.48-fold greater than that of the control (MSB alone). In contrast, the population densities of isolate PpTS-1 were not significantly different in the MSB amended with CFC filtrate from any of the three biocontrol isolates. However, populations were greater (0.3- to 0.6-

Table 2. Mean colony diameters of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) when grown in association with different species of bacteria or fungi.

Treatment	Species	GenBank Accession No.	Antagonism against *Fol*[a]		
			Mycelial growth (mm) of *Fol* in dual culture assays	Class of antagonism[b]	Mycelial growth (mm) of *Fol* in cell free culture filtrate assays
PpTS-1	*Pseudomonas putida*	GU048848	55.6 a	-	36.4 d
BsS2BC-1	*Bacillus subtilis*	AM268039	47.4 c	-	57.7 b
CgCG-2	*Chaetomium globosum*	AY429049	52.3 b	-	44.0 c
ThS17TH	*Trichoderma harzianum*	GUO48855	-	Class I	64.2 a
LSD (*P*=0.05)			3.2	-	0.8

[a] Values are means of three replications.
[b] Isolates that overgrew the pathogen, covering at least two-thirds of the medium surface in dual culture was considered antagonistic (Shanmugam *et al.,* 2013). *Fol* alone served as experimental controls. Treatment means followed by a common letter(s) are not significantly different from each other (LSD tests, $P = 0.05$).

Table 3. Mean population densities or mycelium weights for different potential biocontrol agents when grown in association with other antagonists.

Treatment[a]	Optical densities (O.D.) and population densities for two rhizobacteria[b]				Mycelial weights (g L^{-1}) of two antagonistic fungi[b,d]	
	BsS2BC-1		PpTS-1		ThS17TH	CgCG-2
	O.D. (600 nm)	Population density ($\times 10^8$ cfu mL^{-1})[c]	O.D. (600nm)	Population density ($\times 10^8$ cfu mL^{-1})[c]		
BsS2BC-1-CFC (10%)	-	-	2.4 b	4.7 c	12.3 a	8.5 a
PpTS-1-CFC (10%)	2.9 a	5.7 a	-	-	11.8 b	8.7 a
CgCG-2-CFC (10%)	2.0 b	4.0 b	2.8 a	5.5 b	12.0 a	-
ThS17TH-CFC (10%)	2.1 b	4.2 ab	2.9 a	5.7 a	-	8.2 b
Control (MSB alone)	1.1 c	2.3 c	1.7 c	3.5 d	11.4 ab	8.0 ab
LSD ($P = 0$ .05)	1.7	3.2	1.3	3.2	2.2	1.9

[a]BsS2BC-1, PpTS-1, ThS17TH and CgCG-2, respectively, refer to the biocontrol isolates of *Bacillus subtilis* (GenBank No. AM268039), *Pseudomonas putida* (GenBank No. GU048848), *Trichoderma harzianum* (GenBank No. GU048855) and *Chaetomium globosum* (GenBank No. AY429049).
[b] Values are the means of three replications.
[c] 0.5 OD at 600 nm = 1×10^8 cfu mL^{-1}.
[d] No mutual antagonism was observed among them in dual plates or CFC filtrate assays. Treatment means followed by a common letter(s) are not significantly different from each other (LSD tests; $P = 0.05$).

fold) than the MSB alone (control). The increases in population densities of the rhizobacterial isolates in the MSB amended with CFC filtrate indicated positive interactions with metabolites from the other biocontrol isolates. Unlike the rhizobacterial isolates, though the mycelial weights of isolate ThS17TH in the MSB amended with the CFC filtrate of BsS2BC-1 and CgCG-2 were significantly greater than that of the control (MSB alone), no statistically significant change in mycelial weight was detected for isolate PpTS-1. Likewise, no difference in mycelial weight of isolate CgCG-2 was detected in MSB amended with any of the three other biocontrol isolates. Increased mycelial weight or no change in the mycelial growth indicated positive interactions of the fungal isolates with the metabolites of the other biocontrol isolates (Table 4).

Table 4. Growth inhibition of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) by antagonists upon interaction with other antagonists in cell free filtrate (10%) assays.

Treatment[a]	Inhibition zone (mm)[b]		Mycelial growth of Fol (mm)[b]	Class of antagonism[b]
	BsS2BC-1	PpTS-1	CgCG-2	ThS17TH
BsS2BC-1-CFC	-	9.8 ab	50.2 b	I
PpTS-1-CFC	13.7 a	-	48.7 ab	I
ThS17TH-CFC	9.8 d	10.2 b	52.6 a	-
CgCG-2-CFC	11.9 b	10.2 b	-	I
Control	11.0 c	10.5 a	49.0 ab	I
LSD (P = 0.05)	1.9	1.3	1.6	-

[a] BsS2BC-1, PpTS-1, ThS17TH and CgCG-2, respectively, refer to the biocontrol isolates of *Bacillus subtilis* (GenBank No. AM268039), *Pseudomonas putida* (GenBank No. GU048848), *Trichoderma harzianum* (GenBank No. GU048855) and *Chaetomium globosum* (GenBank No. AY429049).
[b] Values are means of three replications. Treatment means followed by a common letter(s) are not significantly different from each other (LSD tests; P = 0.05).

Mycelial growth and lytic enzyme production by isolate CgCG-2, as affected by the potential biocontrol isolates

In evaluating the effects of live or heat-treated cells of isolates BsS2BC-1 and PpTS-1, or conidia of isolate ThS17TH, mycelial weight of isolate CgCG-2 was similar or greater (0.1- to 0.4-fold) from the treatments than that for the control (MSB alone). Among the treatments, 0.3- to 0.4-fold greater mycelial growth than the control was detected for MSB amended with heat-treated cells of both the rhizobacteria, indicating that the bacterial cells were used as nutrient sources by the biocontrol fungus. No significant differences in mycelial weight over the control were noticed from the treatments of MSB amended with live cells or CFC filtrate of isolate BsS2BC-1 (Table 5). In the enzyme assay, similar to results for mycelial growth, isolate CgCG-2 had greater chitinase (0.5- to 3.0-fold) and β-1,3-glucanase (0.2- to 0.6-fold) activities in the CFC filtrates of MSB amended with the cells/conidia in relation to MSB alone (control). The enzyme activities were greater in the heat-killed cells, with 2.5- to 3.0-fold increases in chitinase activity, and 0.5- to 0.6-fold increases in β-1,3-glucanase activity compared with the MSB control. As observed for the mycelial weight assessments, no statistically significant differences in the enzyme activities were detected for the CFC filtrates of MSB amended with the live cells or the CFC (Table 5).

Chitinase gene expression in isolate CgCG-2 after different treatments of potential biocontrol isolates

In the qRT-PCR assays, expression of the endochitinase gene *chi46* was upregulated (1.1- to 5.1-fold) from all the treatments compared to the MSB control. Among the isolate BsS2BC-1 treatments, *chi46* expression was

Table 5. Mean mycelial growth and lytic enzyme production by *Chaetomium globosum* isolate CgCG-2 upon interaction with other antagonists.

Treatment[a]	Mycelial weight (g) of CgCG-2	Enzyme assays[b]	
		Chitinase (N-acetyl-β-D glucosaminidase) activity (U/mL of culture filtrate)[c]	Beta-1,3- glucanase (U of glucose/mL of culture filtrate)[d]
BsS2BC-1-Live cells	1.6 cd	0.9 b	10.2 b
BsS2BC-1-Heat killed cells	1.9 ab	1.6 a	11.6 ab
PpTS-1-Live cells	1.8 ab	0.9 b	9.8 bc
PpTS-1-Heat killed cells	2.1 a	1.4 a	12.3 a
ThS17TH-CFC (10%)	1.7 cd	0.6 c	10.8 ab
Control (MSB alone)	1.5 cd	0.4 d	7.9 c
LSD (P = 0.05)	0.2	0.4	2.2

[a] BsS2BC-1, PpTS-1, ThS17TH and CgCG-2, respectively, refer to the biocontrol isolates of *Bacillus subtilis* (GenBank No. AM268039), *Pseudomonas putida* (GenBank No. GU048848), *Trichoderma harzianum* (GenBank No. GU048855) and *Chaetomium globosum* (GenBank No. AY429049).
[b] Values are means of three replications; [b] One unit of each enzyme was defined as the amount of enzyme that will release 1.0 μmole of p-nitrophenol from 4-nitrophenyl-N-acetyl-β-D-glucosaminide per min, at pH 4.8 at 37°C.
[c] One unit min^{-1} of beta-1,3-glucanase liberated 1 mg of reducing sugar (measured as glucose) from laminarin, at pH 5.0 and 37°C. Treatment means followed by a common letter(s) are not significantly different (LSD tests (P = 0.05).

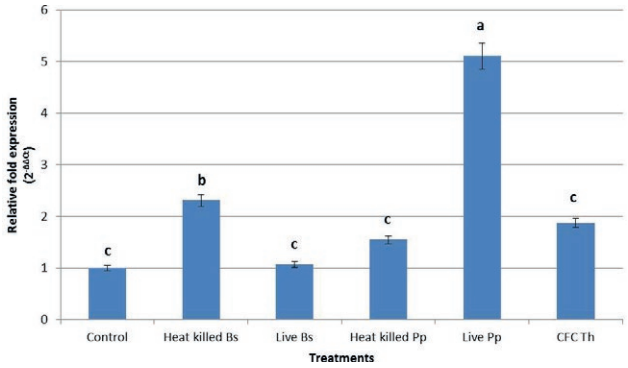

Figure 1. Relative expression of the biocontrol gene *chi46* in CgCG-2 on interaction with other antagonists. Control- MSB, Heat killed Bs- Heat killed cells of *Bacillus subtilis* BsS2BC-1, Live Bs- Live cells of *B. subtilis* BsS2BC-1, Heat killed Pp- Heat killed cells of *Pseudomonas putida* PpTS-1, Live Pp- Live cells of *P. putida* PpTS-1 and CFC Th- 10% CFC of *Trichoderma harzianum* ThS17TH. FOL mycelium (1%) was amended as a carbon source in MSB; Error bars indicate SD ($P < 0.05$). The amounts of cDNA in the samples were normalised by employing *α-tubulin* as an internal control.

greater (2.3-fold) in the MSB-containing heat-killed cells, whereas, among the isolate PpTS-1 treatments, expression was strongly upregulated (5.1-fold) in the MSB inoculated with the live cells, and the expression was greater than any of the other treatments. In the isolate ThS17TH treatment, though expression was upregulated (1.9-fold) in comparison to the control, expression was less (0.2- and 1.7-fold) than that of MSB with heat-killed isolate BsS2BC-1 or live cells of isolate PpTS-1 (Figure 1).

Polyhouse assessments of biocontrol isolates for Fusarium wilt control

In evaluation of cell suspensions of the potential biocontrol isolates for growth promotion and vascular wilt reduction in tomato, after inoculations with the fungal pathogen, the biocontrol treatments of seed or soil generally increases tomato plant height relative to pathogen controls by 3.5 to 135.4%. Among the biocontrol treatments, except for isolate PpTS-1, the consortia treatments comprising a maximum of three isolates gave the greatest plant heights (101 to 135% increases), and greatest vascular wilt reductions (57% to 71%) compared with the pathogen controls. The isolate CgCG-2 based consortium of PpTS-1 + CgCG-2 + ThS17TH increased plant height by 135%, and gave significantly less (71%) incidence of vascular wilt, compared with the pathogen control. Biocontrol performance of the consortium comprising all four isolates was less than that from the

Table 6. Polyhouse evaluation of biocontrol agents in management of Fusarium wilt of tomato.

Treatment (challenged with *Fol*)[a]	RT+ST (cm)[b]	Vascular wilt (PDI) of tomato[c]
BsS2BC-1	40.7 c	41.7 bc
PpTS-1	60.5 b	25.0 ef
ThS17TH	31.3 d	39.6 bc
CgCG-2	31.1 d	35.4 cd
BsS2BC-1+PpTS-1+CgCG-2	61.6 ab	25.0 ef
BsS2BC-1+PpTS-1+ThS17TH	60.3 b	21.9 ef
BsS2BC-1+CgCG-2+ThS17TH	63.3 ab	22.9 ef
PpTS-1+CgCG-2+ThS17TH	70.7 a	16.7 f
BsS2BC-1+PpTS-1+ThS17TH+CgCG-2	59.5 b	29.2 de
Carbendazim (0.1%)	33.7 cd	45.8 b
Pathogenic control	30.1 d	58.3 a
LSD (P=0.05)	0.6	1.6

[a] BsS2BC-1, PpTS-1, ThS17TH and CgCG-2, respectively, refer to the biocontrol isolates of *Bacillus subtilis* (GenBank No. AM268039), *Pseudomonas putida* (GenBank No. GU048848), *Trichoderma harzianum* (GenBank No. GU048855) and *Chaetomium globosum* (GenBank No. AY429049).-

[b] Mean of 12 replications; RT + ST, root length + shoot length. No wilt incidence was observed in the uninoculated experimental controls. Treatment means followed by a common letter(s) are not significantly different (LSD tests, $P = 0.05$).

[c] The wilt development on each tomato plant was rated as described (Sidharthan *et al.,* 2018): 0 = no symptoms; 1 = <25% of leaves with symptoms; 2 = 26–50% of leaves with symptoms; 3 = 51–75% of leaves with symptoms; 4 = 76-100% of leaves with symptoms. The per cent disease index was calculated as follows: Disease index = (Σ (rating × number of plants rated)/Total number of plants × highest rating) × 100.

mixtures containing three isolates. Among the single-isolate treatments, PpTS-1 gave the greatest increase in plant height (101%) and greatest disease reduction (57%) compared with the pathogen control. However, the plant growth promotion and disease reductions were less than that of the best-performing isolates PpTS-1 + CgCG-2 + ThS17TH consortium. The fungicide treatment was less effective for vascular wilt control and plant growth promotion, giving 21% less wilt and 11% increase in plant height, than the pathogen control (Table 6; Supplementary Figure 6).

Induction of resistance in tomato plants by the potential bioconsortium

To elucidate induction of resistance to vascular wilt in tomato plants by the potential bioconsortia identified in the pot experiments, expressions were assessed of the

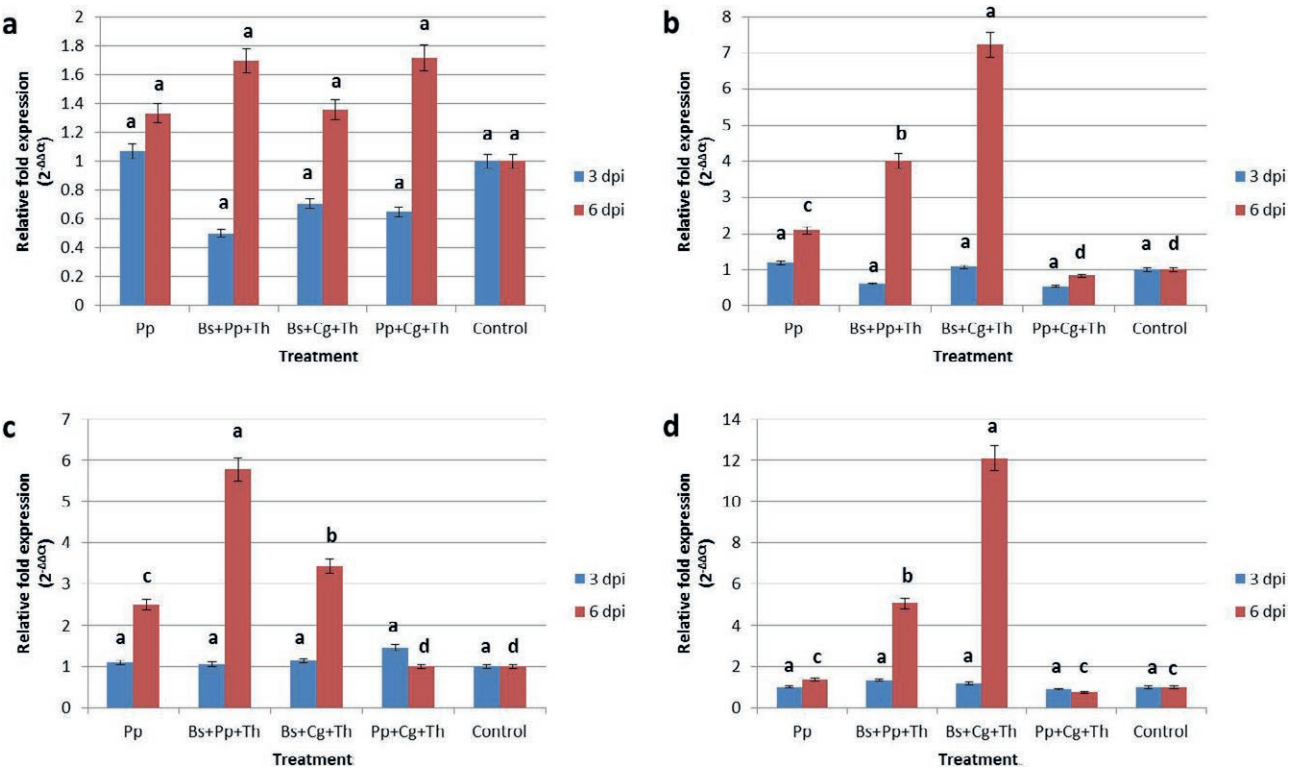

Figure 2. Relative expression of defence genes in tomato plants 'Pusa Rohini' (a) *AOC*, (b) *PR3b*, (c) *PR5* and (d) *gluB*. *AOC* is a gene involved in JA biosynthetic pathway (Uppalapati *et al.,* 2005); *PR3b* and *PR5* were identified as marker genes for Systemic Acquired Resistance, SAR (Shavit *et al.,* 2013; Sotoyama *et al.,* 2016); *gluB* was identified as a marker gene for ET modulated pathway (Martínez-Medina *et al.,* 2013). Bs-*Bacillus subtilis* BsS2BC-1, Pp-*Pseudomonas putida* PpTS-1, Th-*Trichoderma harzianum* ThS17TH, Cg-*Chaetomium globosum* CgCG-2 and Control- Pathogenic control; Error bars indicate SD ($P < 0.05$). The amounts of cDNA in the samples were normalised by employing cyclophilin (*cyp*) as an internal control. Blue and red bars indicate samples obtained at 3- and 6-days post pathogen inoculation, respectively.

JA pathway gene *AOC*, and three defence genes, *PR3B*, *PR5* and *gluB* using qRT-PCR assays. At 3 dpi, in comparison to the pathogen control, not significantly greater expressions of *AOC* were observed for any of the biocontrol treatments. However, at 6 dpi, strong expression (1.0- to 1.7-fold) of *AOC* in comparison to that from the control, was observed for all the bioconsortium treatments (Figure 2). Expressions of defence genes modulated by the SA and ET pathways were increased after the biocontrol treatments. Increases for the SA modulated pathway were 2.1- to 7.2-fold for *PR3b* and 2.5- to 5.8-fold for *PR* for treatments with isolate PpTS-1, or the consortia of isolates BsS2BC-1 + PpTS-1 + ThS17TH or isolates BsS2BC-1 + CgCG-2 + ThS17TH. On the contrary, the best-performing treatment PpTS-1 + CgCG-2 + ThS17TH at 6 dpi reduced expression. At 6 dpi, the consortia of isolates BsS2BC-1 + PpTS-1 + ThS17TH and BsS2BC-1 + CgCG-2 + ThS17TH gave greatest expression of the genes *PR3b* (7.2-fold) and *PR5* (5.8-fold). Similarly, *gluB*, the marker gene for the ET modulated pathway, was significantly overexpressed at 6 dpi from the consortia treatments with isolates BsS2BC-1 + CgCG-2 + ThS17TH or isolates BsS2BC-1 + PpTS-1 + ThS17TH, in comparison to the pathogen control. However, *gluB* was down-regulated from the best-performing treatment, isolates PpTS-1 + CgCG-2 + ThS17TH (Figure 2). Induction of the pathway or defence genes for induced resistance indicated that the reductions in vascular wilt by the biocontrol isolates could be partly plant mediated.

DISCUSSION

In biocontrol of plant diseases, success depends on availability of consortia of effective isolates with varying modes of action and potential to colonise different niches. Both the rhizobacterial isolates BsS2BC-1 and PpTS-1, and the *T. harzianum* isolate ThS17TH used in this study were reported to produce lytic enzymes (Shanmugam and Kanoujia, 2011; Shanmugam *et al.,*

2011b; Shanmugam *et al.,* 2013), whereas isolate CgCG-2 produced antibiotics (Aggarwal *et al.,* 2004; Rashmi *et al.,* 2007). Though the colonizing potential of the biocontrol isolates was not assessed in the present study, the rhizobacteria and fungus biocontrol agents are known to occupy different or complementary niches (Larkin and Fravel, 1998). The bacterial and the *T. harzianum* isolates are potential bioagents when used alone or in combinations for management of Fusarium wilt of tomato or in other crop plants (Shanmugam and Kanoujia, 2011; Shanmugam *et al.,* 2011b; Shanmugam *et al.,* 2013). Testing consortia of these bioagents with the *C. globosum* isolate CgCG-2, another potential bioagent (Aggarwal, 2015) but not previously assessed on tomato, would offer potential benefits, because of the mechanisms outlined above. However, development of a consortium for large-scale applications, needs careful selection and testing of the bioagents for their compatibility, particularly relating to mutual growth inhibition, antagonism against *Fol*, and biocontrol gene expression, as well as validating their biocontrol abilities. The *in vitro* studies outlined here demonstrated compatibility of the biocontrol isolates with the parameters assessed.

Ability of the candidate biocontrol isolates to inhibit *F. oxysporum* f. sp. *lycopersici* in the DC and the CFC filtrate assays indicated their potential efficacies against the pathogen. *In vitro* inhibition of the pathogen by rhizobacterial and *Trichoderma* isolates has been previously reported (Shanmugam *et al.,* 2011b; 2015). The lack of mutual antagonism among the candidate antagonists in either the DC or CFC assays in the present study indicated their compatibility, as well as the suitability of using their cells or metabolites as possible treatments to assess positive interactions for antagonism, host growth increases and antifungal gene or enzyme expressions.

The positive interactions among the biocontrol isolates were also reflected in their antagonistic abilities against *Fol*. Each of the candidate biocontrol isolates displayed greater or similar mycelial growth inhibition of the pathogen in the DC assays, even in the presence of the CFC filtrates of the other potential agents compared with controls without the CFC filtrate. Likewise, in the co-cultivation studies, the CFC filtrates of each of the biocontrol isolates exerted positive interactions with the other isolates, as the bacterial and fungal isolates increased population densities or increased mycelial masses compared to the untreated controls. These results indicate enhanced reliability of the isolates as consortia.

Chitinases and β-1,3-glucanases are potential lytic enzymes elaborated by biocontrol microbes. These enzymes play significant roles in controlling fungal diseases, by degrading chitin and β-1,3-glucan, the chief components of fungal cell walls (Shanmugam *et al.,* 2013; Sharma *et al.,* 2018). Because of the compatibilities identified among the biocontrol isolates, the rhizobacteria and the *Trichoderma* isolates were differentially treated and tested against the *C. globosum* isolate CgCG-2. The CgCG-2 isolate gave greater mycelial growth and lytic enzyme production than the MSB control. Synergism between metabolites of *Pseudomonas* with that of the lytic enzymes of *Trichoderma* in antifungal activity has been previously reported (Woo *et al.,* 2002; Dugassa *et al.,* 2021). Among the treatments, increased mycelial growth and lytic enzyme production were observed for MSB amended with heat-treated cells of both of the rhizobacteria. This indicates the degrading ability of isolate CgCG-2 to use the bacterial cells as its nutrients. Increased production of NAGases, proteases and muramidase to degrade heat-inactivated bacterial cells has been previously reported for different species of *Trichoderma* (Manczinger *et al.,* 2002; Shanmugam *et al.,* 2013). In comparison to heat-treated cells, enzyme production was reduced in the differentially treated bacterial treatments. From an ecological perspective, the CgCG-2 isolate is not supposed to produce high amounts of lytic enzymes to compete with the bacteria for nutrition on co-cultivation. Hence, the relatively lesser production of lytic enzymes from the bacterial treatment could be a positive interaction.

Gene expressions have been extensively studied during interactions of biocontrol isolates. In the co-cultivation of a wild *P. fluorescens* isolate with that of *T. atroviridae,* though a significant reduction in the growth of the fungus was observed, *P. fluorescens* had a positive biocontrol gene expression influence on *T. atroviridae* (Lutz *et al.,* 2004). Similarly, upregulation of secondary metabolite genes in *T. asperellum* was observed upon co-cultivation with *B. amyloliquefaciens* (Karuppiah *et al.,* 2019). The endochitinase gene, *chi46* has been shown to play a major role in the antagonistic ability of *C. globosum* (Liu *et al.,* 2008). Hence, its expression in isolate CgCG-2 isolate was assessed during its growth in differentially treated bacterial cells or fugus conidia. Upregulation of the gene to various levels from all treatments compared to the MSB control indicated positive interactions of the CgCG-2 isolate with that of the other biocontrol isolates. Therefore, the *in vitro* studies showed that each of the four candidate biocontrol isolates was compatible with the others, so these isolates were assessed for vascular wilt control in the polyhouse experiments.

In evaluating the biocontrol efficacies of the isolate mixtures designed based on the *in vitro* experiments, the suspension cultures of the consortia as seed soil treat-

ments reduced vascular wilt incidence and promoted tomato plant growth, in comparison to the pathogen controls. Among the treatments, the isolate CgCG-2 containing consortium of isolates PpTS-1 + CgCG-2 + ThS17TH increased plant height to a greater extent, and gave less vascular wilt incidence, than the pathogen controls. Since the polyhouse provided similar conditions to those applying in fields, this isolate mixture could be suitable for field studies, to assess its applicability for practical biocontrol of vascular wilt. Disease suppression and growth-promoting abilities of biocontrol consortia have been demonstrated for other diseases (Kamalakannan and Shanmugam, 2009; Senthilraja *et al.,* 2010). In the polyhouse experiments of the present study, since the isolate mixture gave better biocontrol under the pathogen challenge, the inoculated plants may have been primed to activate their defence mechanisms. For this reason and to understand the defence mechanisms underlying the biocontrol ability of isolate CgCG-2, the roles of marker defence genes were assessed.

In biocontrol of plant diseases, the bioagents act directly on pathogens and/or through induction of resistance in host plants. Resistances may be systemically induced in plants upon elicitation by pathogens. Specific isolates of non-pathogenic PGPR or fungi induce, respectively, Systemic Acquired Resistance (SAR) or Induced Systemic Resistance (ISR). ISR is characterized by defence priming, where the defence responses are rapidly induced than pathogen attack-inducing resistance, which is metabolically less costly to host plants. Induction of resistance in plants involves hormones, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Pieterse *et al.,* 2009), to activate defence responses. Among the defence responses, the SA pathway commonly induces pathogenesis-related proteins during SAR. In contrast, JA/ET induces proteinase inhibitor (PI), polyphenol oxidase (PPO), arginase, threonine deaminase, leucine aminopeptidase and acid phosphatase during ISR (Shanmugam and Kanoujia, 2011). However, enhanced expression of SA pathway genes by biocontrol treatments, as well as JA/ET pathway genes, has also been reported (Morán-Diez *et al.,* 2012; Kamou *et al.,* 2020). Hence, to elucidate induction of resistance in tomato plants by the potential bioconsortia identified in the pot experiments of the present study, expression was assessed of the JA pathway gene *AOC*, the marker genes *PR3b*, and *PR5* for the SA modulated pathway, and an ET modulated pathway gene, *gluB*, for the best performing biocontrol treatments (Uppalapati *et al.,* 2005; Martínez-Medina *et al.,* 2013; Shavit *et al.,* 2013; Sotoyama *et al.,* 2016).

The SA pathway involves endogenous accumulation of SA and the defence regulatory protein NPR 1 to activate PR genes. Among the PR genes, chitinases accumulate in plants to degrade the cell wall components of fungal pathogens, as part of self defence mechanisms (Shanmugam, 2005). Among chitinases, *PR3b* has been shown to have fungitoxic action (Woloshuk *et al.,* 1991; Sun *et al.,* 2019). Like the chitinases, the *PR5* gene encoding thaumatin-like proteins is known to exhibit antimicrobial activity (Shavit *et al.,* 2013). In the present study, among the pathway genes, strong expression of *AOC* compared to that of for control treatments was observed for all the biocontrol treatments. In further studies on the relative expressions of *PR3b* and *PR5*, though the genes were strongly expressed in the treatments, the combination treatments with isolates BsS2BC-1 + CgCG-2 + ThS17TH and BsS2BC-1 + PpTS-1 + ThS17TH reduced expression compared with the experimental controls. The best performing treatment was that with isolates PpTS-1 + CgCG-2 + ThS17TH. Similar to *PR3b* and *PR5*, the combination treatments with isolates BsS2BC-1 + CgCG-2 + ThS17TH and BsS2BC-1 + PpTS-1 + ThS17TH showed strong expression of *gluB*, which was significantly greater than that from the pathogen controls. However, *gluB* was downregulated in the best-performing treatment with isolates PpTS-1 + CgCG-2 + ThS17TH. Despite the down-regulation of selected defence genes at 3 dpi and 6 dpi, involvement of other tomato defence genes is likely to have contributed to the enhanced performance of the PpTS-1 + CgCG-2 + ThS17TH combination.

Induction of the pathway or defence genes for induced resistance indicated that the reduction in vascular wilt control by the biocontrol isolates could be partly plant-mediated. Previous research has shown that induction of plant defence genes, chitinase, and β-1,3-glucanase can lead to decreased disease incidence (Shanmugam *et al.,* 2011b; 2015). In defence priming, the defence responses are more rapidly induced upon pathogen attack. The lack of early expression of genes (at 3 dpi) observed in the present study requires further investigation.

Beside disease suppression, the biocontrol treatments also promoted the tomato plant growth. This could be due to suppression of pathogens, increased availability of nutrients, or production of plant growth-promoting substances. Nevertheless, elicitation of the plant defence genes observed in this study indicated that the induction of host defence could also be a mechanism for growth promotion.

The *C. globosum*-based microbial consortia could be important for subsistence farming of tomatoes grown with limited rotations, where soilborne diseases can severe problems, fungicide treatments are unaffordable. The consortia identified in the present study have poten-

tial as inputs in integrated disease management systems, once they have been formulated and tested under field conditions. However, consortium efficacy in the field is often primarily determined by the ecological processes (Verma *et al*., 2007; Niu *et al*., 2020). Therefore, effects of the ecological factors on the bioagents in a consortium must be assessed for successful utilisation of isolate mixtures. Unlike most current biocontrol studies, which mainly assess disease incidence at a particular times, temporal dynamics must be evaluated. More research is also required to elucidate associations of biocontrol efficacy and variability with the microbial community structure where the biocontrol action takes place. Careful consideration of these factors is likely to favour field success of identified biocontrol consortia.

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