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Biological management of vascular wilt of tomato caused by *Fusarium oxysporum* f.sp. *lycospersici* by plant growth-promoting rhizobacterial mixture

Veerubommu Shanmugam *[,1], Nandina Kanoujia [1]

Floriculture Pathology Laboratory, Institute of Himalayan Bioresource Technology, Council of Scientific and Industrial Research, Palampur, 176 061 Himachal Pradesh, India

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ABSTRACT

Two plant growth promoting rhizobacterial (PGPR) strains, GIBC-Jamog (*Bacillus subtilis*) and TEPF-Sungal (*Burkholderia cepacia*) and PGPR strain mixtures, S2BC-1 (*B. subtilis*)+GIBC-Jamog (*B. subtilis*) and S2BC-2 (*Bacillus atrophaeus*)+TEPF-Sungal (*Burkholderia cepacia*) which inhibited the mycelial growth of vascular wilt fungus *Fusarium oxysporum* f.sp. *lycopersici* were selected for assessing vascular wilt control in tomato by localized and induced systemic resistance (ISR) experiments in the greenhouse. Seed bacterization and soil application of S2BC-1+GIBC-Jamog challenge-inoculated with *F. oxysporum* f.sp. *lycopersici* resulted in significantly lower incidence (54.9% and 53.8% reduction) relative to the non-bacterized pathogen control in localized and split-root experiments, respectively. In localized studies, the lower disease incidence coincided with increases in the plant vigor index and fresh weight of 44.1% and 183.4%, respectively, relative to the pathogen control. Analysis of root samples in localized and split-root experiments also indicated increased induction of chitinase (2.2- and 2.6-fold, respectively) and β-1,3-glucanase (1.4- and 1.1-fold, respectively). In native gel activity assay, S2BC-1+GIBC-Jamog with challenge-inoculation, expressed high intensity peroxidase isoforms in localized and ISR experiments. It is presumed that the induced chitinase, β-1,3-glucanase and peroxidase in localized and split-root experiments may be involved in the reduction of vascular wilt development in tomato. Reduced disease severity coupled with enhanced enzyme production elicited by S2BC-1+GIBC-Jamog in localized and split-root experiments indicate that its mode of action for vascular wilt suppression in tomato is through both direct biocontrol and ISR.

1. Introduction

Tomato (*Lycopersicon esculentum* Mill) is an important vegetable crop, whose cultivation is often hampered by vascular wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* (Synder and Hans). Chemical control measures create imbalances in the microbial community, which may be unfavorable to the activity of the beneficial organisms and may also lead to the development of resistant strains of pathogen. Developing resistant varieties can be difficult in the absence of dominant genes and development of new races of the pathogen overcoming host resistance. Biocontrol by use of plant growth promoting rhizobacteria (PGPR) represents a potentially attractive alternative disease management approach since PGPR are known for growth promotion and disease reduction in crops (Jetiyanon and Kloepper, 2002). However, application of a single biocontrol agent often results in inconsistent field performance as it is less likely to be active in different soil environment and agricultural ecosystems (Raupach and Kloepper, 1998). Combined application of two or more biocontrol strains is likely to more closely mimic the natural situation and may, therefore, represent a more viable control strategy (de Boer et al., 1999). The level and consistency of control are therefore greatly enhanced due to multiple modes of action, a more stable rhizosphere community, and effectiveness over a wider range of environmental conditions (Larkin and Fravel, 1998).

Disease reduction by PGPR in colonization of plant roots occur directly, through competition for space, nutrients and ecological niches or production of antimicrobial substances, and indirectly, through induction of systemic resistance (ISR) (Kloepper and Beauchamp, 1992; Liu et al., 1995). Induced resistance is a state of enhanced defensive capacity developed by a plant reacting to specific biotic or chemical stimuli (van Loon et al., 1998). Disease reduction by induction of resistance has been accounted for by a large number of defence enzymes, including peroxidase (PO) and polyphenol oxidase (PPO) that catalyze the formation of lignin and phenylalanine ammonia-lyase (PAL) that is involved in phytoalexins and phenolics bio-synthesis. The pathogenesis-related proteins (PRs) such as chitinase (PR-3 family) and β-1,3-glucanase (PR-2 family) are the other defence enzymes which accumulate following pathogen attack and degrade the fungal cell wall by lysis.

* Corresponding author. Fax: +91 1894 230433.
E-mail address: shanpatho@yahoo.com (V. Shanmugam).
[1] These authors contributed equally to this research.

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Chitin and glucan oligomers released during degradation of fungal cell wall act as elicitors that stimulate various defence mechanisms in the plants (Frindlender et al., 1993). The induced protection by selected strains of PGPR has been shown to promote plant growth as well (Ramanathan et al., 2002; Ramamoorthy et al., 2002; Nandakumar et al., 2001).

Among PGPR, fluorescent pseudomonads have been reported to be effective against a broad spectrum of plant pathogens, including fungi, bacteria and viruses in many plant species, *e.g.* bean, carnation, cucumber, radish, tobacco, tomato and the model plant *Arabidopsis thaliana* (van Loon et al., 1998). Similarly, the sporulating Gram-positive bacteria like *Bacillus* spp. have also been used successfully for plant disease control (Kloepper et al., 2004).

In plant-pathogen interactions, though ISR by PGPR is well documented (van Loon et al., 1998; Kloepper et al., 2004), little is known about the ability of a strain mixture to reduce disease incidence both locally and systemically through induction of host defence enzymes. The present study therefore assessed the efficacy of *in vitro* selected rhizobacterial mixtures along with individual strains on vascular wilt reduction in tomato in both localized and induced systemic resistance experiments under greenhouse conditions. The role of defence enzymes in disease suppression by the biocontrol agents was also ascertained.

2. Materials and methods

2.1. Fungal pathogen and inoculum preparation

F. oxysporum f.sp. *lycopersici* (FOL) isolate TOFOL-IHBT (GenBank Acc. No. HM484352) causing vascular wilt of tomato was isolated from infected tissues on Potato Dextrose Agar (PDA) medium. Pathogenicity tests for the fungus were carried out in artificial inoculated conditions (Shanmugam et al., 2009). The pathogen was re-isolated from the diseased tissues to confirm Koch's postulates.

To produce pathogen inoculum, the fungus was cultured on PDA for 7 days in Petri plates. The microconidial suspension was prepared by pouring 20 mL of sterile distilled water in each Petri plate. The concentration of microconidia was adjusted to 1000 - conidia/mL.

2.2. PGPR strains and inoculum preparation

The rhizobacterial strains were obtained from the culture repository of Floriculture Pathology Laboratory of the host institute (Table 1). For inoculum preparation, a single colony of the fluorescent pseudomonads or *Bacillus* spp. was inoculated in King's broth B or nutrient broth, respectively, and grown for 48 h with constant shaking (180 rpm) at 28 ± 2 °C. The culture obtained at stationery phase was centrifuged at 6000 rev/min for 10 min and the bacterial cells were resuspended in phosphate buffer (100 mM; pH 7.0). The cell concentration was adjusted to 3×10^8 CFU/mL (0.3 OD at 595 nm = 10^8 CFU/mL).

2.3. Evaluation of PGPR for antagonism

The antifungal activity of the rhizobacteria was assessed against FOL by dual culture technique on PDA in triplicate with or without supplementation of $FeCl_3$ (100 μg/mL). PDA plates inoculated with the pathogen alone were maintained as control. The plates were incubated at 28 ± 2 °C for 7 days and the inhibition zone was measured from the edge of mycelium to the bacterial streak, when the control plates showed full growth. The rhizobacterial strains showing the greatest inhibition were used for further studies.

2.4. Elucidation of functional traits of antagonistic PGPR

The antagonistic strains were screened by plate assays for the production of the following functional traits: hydrogen cyanide, HCN (Verma et al., 2007); phosphate solubilization (Pikovskaya, 1948) and indole acetic acid, IAA (Bric et al., 1991) for plant growth promotion and siderophore (Schwyn and Neilands, 1987) and chitinase (Wen et al., 2002) for antifungal activity. Briefly, the production of HCN was determined by growing the bacterial isolates in tryptic soy broth. Filter paper (Whatman No. 1) strips saturated with alkaline picrate solution were placed inside the conical flasks in a hanging position. Sodium picrate present in the filter paper was observed for a change in color after 48 h. For IAA, single colonies from cultures that were grown overnight were streaked onto LB agar and the plates overlaid with sterile Whatman No. 1 filter paper. After incubation for 3 days, the paper was removed and treated with Salkowski's reagent. IAA production was identified by the formation of a red halo on the paper immediately surrounding the colony. To detect the phosphate solubilizing bacteria, the strains were streaked onto Pikovskaya's agar medium. Strains that induced clear zone around the colonies after 3 days were considered as positive. Siderophore production was determined after 3 days by the chrome azurol S agar assay on the basis of change in color of the medium from blue to orange. The chitinase activity of strains was tested on chitin agar medium, and the chitinase activity was identified by clear zone around the cells after 5 days. All these assays were replicated thrice for each of the isolates.

2.5. Efficacy of in vitro selected antagonists on plant growth-promotion

For bacterization, seeds of tomato (cv. Solan Vajr) were surface sterilized with 1% sodium hypochlorite for 1 min and soaked in

Table 1
Antagonistic rhizobacteria selected against *F. oxysporum* f.sp. *lycopersici*.

Bacterial strain	Identity	GenBank accession No.	Zone of mycelial growth inhibition (mm)[a] in dual culture assay	
			Without $FeCl_3$	With $FeCl_3$
TEPF-Sungal	*Burkholderia cepacia*	GUO48848	12	12
XXPF-MDU(2)	*Burkholderia cepacia*	GUO48852	10	10
XXPF-MDU(1)	*Pseudomonas fluorescens*	GUO48851	8	9
BGPF-Nagrota	*Pseudomonas putida*	GUO48847	10	10
LSD (P = 0.05)			1.9	1.9
CABS-IHBT	*Bacillus subtilis*	AM 265566	13	12
S2BC-1	*Bacillus subtilis*	AM268039	9	10
S2BC-2	*Bacillus atrophaeus*	AM 268040	10	10
GIBC-Jamog	*Bacillus subtilis*	GUO48875	12	11
GIBC-Kiari(1)	*Bacillus thuringiensis*	GUO48876	8	8
LSD (P = 0.05)			1.8	1.8

[a] Mean of three replications.