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Induced Systemic Resistance by *Bacillus vallismortis* EXTN-1 Suppressed Bacterial Wilt in Tomato Caused by *Ralstonia solanacearum*

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Biocontrol activity of five strains of selected rhizobacteria were tested in tomato against bacterial wilt caused by *Ralstonia solanacearum*. After root bacterization the plants were grown in a perlite-hydroponic system. Upon challenge inoculation with the pathogen, all of the rhizobacterial strains efficiently suppressed the bacterial wilt in tomato in various rates, at maximum by the strain, *Bacillus vallismortis* strain EXTN-1. While the percent of infected plants in the non-bacterized control plants were 95%, it was only 65% in plants pre-treated with EXTN-1. It was also demonstrated that the movement of *R. solanacearum* within the stem was significantly hampered when the plants were root bacterized. As EXTN-1 has no antagonistic properties against *R. solanacearum*, the bacterial wilt was probably suppressed by a mechanism other than antibiosis. Previously, the strain had been proven to produce an efficient elicitor for inducing systemic resistance in many crops. As the present study confirmed that EXTN-1 has the ability for reducing the pathogen spread in tomato, the strain could be effectively used as a potential biocontrol agent against bacterial wilt.

Keywords **:** *Bacillus vallismortis* EXTN-1, Bacterial wilt, bicontrol, induced systemic resistance

Bacterial wilt caused by *Ralstonia solanacearum* is a disease, widely distributed in tropical, subtropical and temperate regions world wide (Hayward, 1991). Various strategies for controlling this disease have been developed including breeding cultivars with resistance genes, improvement of cropping systems, soil amendments, and biological control (Frey et al., 1994; Trigalet et al., 1998; Guo et al., 2004). As an inevitable component on the Integrated Disease Management (IDM), biological control reduces the dependence on high-risk chemicals for disease management and is environment friendly (Alström, 1991; Anith et al., 2004).

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The restricted growth of *R. solanacearum* in suppressive soils has been demonstrated (Shiomi et al., 1999). The various mechanisms by which the rhizobacterial strains impart disease resistance has been proposed. Nakaho et al. (2000) suggested that reduced movement and multiplication of *R. solanacearum* within the tomato stem was caused by host resistance. It is worth investigating the population dynamics of the pathogen in rhizobacteria-treated tomato stems, as they have proved to be resistance-inducing agents in host plants.

In the present study five different strains of rhizobacteria were tested for their efficiency to suppress bacteria wilt of tomato. The strains had been proven as antagonists against many of the plant pathogens *in vitro*. One of the strains, *Bacillus vallismortis* strain, EXTN-1 was originally isolated from red pepper and has been proven as an efficient plant growth promoting and disease controlling agent in many different crops including rice, potato, cucumber, tomato and tobacco against multiple pathogens (Park et al., 2006a, 2006b). Multiple mechanisms were found to be involved in EXTN-1 mediated disease control in plants: antibiosis and induced systemic resistance. The present investigation also included studies on the effect of rhizobacterial ISR in reducing pathogen accumulation within host tissues.

Materials and Methods

Bacterial strains. The strains of bacteria used in this study had been short listed from a collection of over 500 strains of rhizobacteria, based on their potential to inhibit the growth of various pathogens *in vitro*. The strains were identified by fatty acid analysis as *Bacillus vallismortis* strain EXTN-1, *B. subtilis* strain 816-6, *B. pumilus* strain 228-7, *Bacillus* sp. 113-3 and *Paenibacillus polymixa* strain H32-5. These strains were grown in tryptic soy agar (TSA) and cell suspensions were prepared in 10 mM $MgSO_4$ after scraping it out from the agar plates. The bacterial wilt pathogen, *R. solanacearum* was multiplied in sucrose peptone broth (SPB) and cells pelleted by centrifugation to suspend in 10

mM $MgSO_4$.

All experiments in the current paper were repeated twice and data were statistically analyzed by ANOVA and means were compared with LSD at P=0.05.

***In planta* studies on disease suppression.** The tomato *Lycopersicum esculentum cv.* Koko seeds were surface sterilized using 70% ethyl alcohol (2 min) and 1% sodium hypochlorite (2 min) and washed four times in sterile water. The seeds were germinated in wet nylon sponge. Upon a week of growth, the plants were carefully pulled out and dipped in the rhizobacterial suspension for 30 min and planted in a perlite-hydroponic system. The plants were allowed to grow for 15 days. The plants were challenged at the rhizosphere with a log-6 suspension of *R. solanacearum*. The plants were observed for disease symptom development.

Population dynamics of *R. solanacearum* within tomato stem. Another set of plants as above were set up and after 20 days of challenge inoculation, the stem were collected and cut into 1 cm segments from bottom to top until 10 cm. These segments were surface sterilized as described above (for tomato seeds) and macerated aspetically in pulverize dilutions of the above were made in 10 mM $MgSO_4$ and plated in TSA with triphenyl tetrazolium chloride (TTC). The plates were incubated at 26°C for 3 days to observe the colonies of *R. solanacearum*.

Antibiosis by EXTN-1 against *R. solanacearum*. EXTN-1 was tested against *R. solanacearum in vitro* to find whether there is an antagonistic response. The assay was done in TSA. The cell suspension of *R. solanacearum* was spread plated on to TSA and 5 µl of cell suspension of EXTN-1 was spotted at four different places in the medium and the plates were incubated at 26°C for 48 h. The plates were observed for an antagonistic interaction between the bacteria.

Results

***In planta* studies on disease suppression.** The plants under different rhizobacterial treatment did not develop disease symptoms within 12 days after challenge with the pathogen, while 15% of the untreated control plants developed significant wilt symptoms. Five to fifteen percent of plants under rhizobacterial treatment developed disease symptoms in 20 days while 85% of untreated challenged plants were infected. Nearly all (95%) of control plants were infected by 30 days after challenge (DAC) when only 65% of plants infected in the plants treated with EXTN-1 (Table 1, Fig. 1). Even though the strain 111-3 protected the plants significantly until 20 DAC, the protection was not prolonged as many of these plants became infected by 30 DAC. The protection offered by EXTN-1 was found to be steady over the period of time. When nearly all non-bacterized control plants were infected with prominent bacterial wilt symptoms, only 65% of the plants under EXTN-1 treatment were infected, at the end of study period.

Table 1. The ability of different strains of bacteria in disease suppression

Treatments (Bacterial strains)	Percent plants affected by bacterial wilt disease*		
	12 DAC**	20 DAC	30 DAC
816-6	0.0 b	15.0 d	80.0 b
228-7	0.0 b	50.0 b	75.0 b
111-3	0.0 b	5.0 e	90.0 a
H32-5	0.0 b	25.0 c	80.0 b
EXTN-1	0.0 b	15.0 d	65.0 c
Control	15.0 a	85.0 a	95.0 a

*Values in a column, corresponding to same letters do not differ significantly at $P = 0.05$

**DAC: Days After Challenge (inoculation with *R. solanacearum*)

Fig. 1. EXTN-1 mediated wilt suppression in tomato.

All the strains except 111-3 showed significant levels of disease suppression against *R. solanacearum*. Strain EXTN-1 was found to be the best in protecting the plant from bacterial wilt. There was significant level of disease suppression when the plants were pre-treated with *B.*

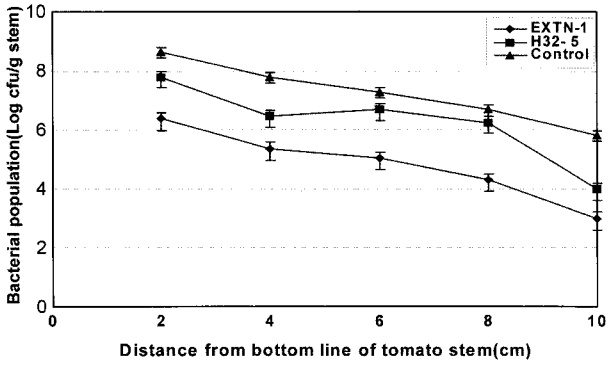

Fig. 2. Population density of *Ralstonia solanacearum* in different treatments at different lengths from the collar region of tomato.

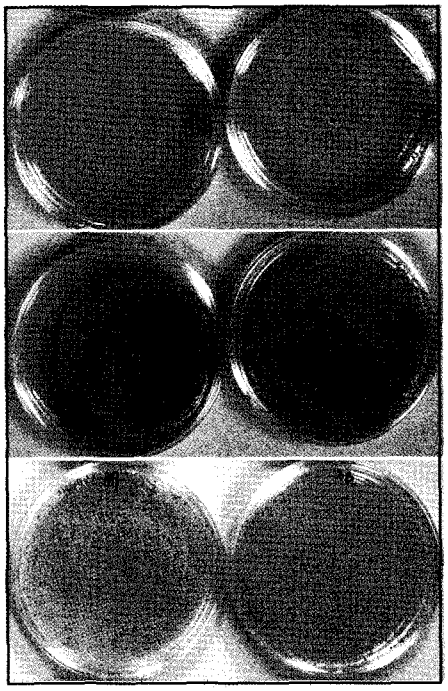

Fig. 3. Population of *Ralstonia solanacearum* in tomato stems. The plants had been pre-treated with rhizobacterial strains, EXTN-1 and H32-5 and challenge inoculated with *R. solanacearum*. Non-bacterized but challenge inoculated control plants had also been maintained. The population of *R. solanacearum* in the stem was enumerated on TSA amended with TTC after serial dilution of each sample. The colonies of *R. solanacearum* appeared red. The figure shows plates inoculated from same dilutions from similar regions of stem cuttings.

vallismortis EXTN-1.

Population dynamics of *R. solanacearum* within the tomato stem. When the lowest stem of the control plants harbored a population of 9.1×10^8 cfu of *R. solanacearum* per gram of tissue, the similar portion of stem of EXTN-1 treatment had only 8.4×10^5 cfu/g of *R. solanacearum*. Obviously, the population of *R. solanacearum* decreased with increasing height of stem. The details of population dynamics of the pathogen in different treatments have been described in Fig. 2 and Fig. 3.

Fig. 4. Antibiosis test between EXTN-1 (A) and *Ralstonia solanacearum* (B). EXTN-1 was not found to be antagonistic to *R. solanacearum*.

Antibiosis by EXTN-1 against *R. solanacearum*. The *in vitro* assay showed that there is no antibiosis caused by EXTN-1 against *R. solanacearum* (Fig. 4).

Discussion

Results presented in this paper clearly demonstrated the disease suppressive potential of strains of rhizobacteria, EXTN-1 being the best among the five. All of the tested strains protected the plants from developing wilt symptoms till the first 12 days after challenge inoculation with the bacterial pathogen. Five to fifty percent of the rhizobacteria-treated plants showed disease symptoms upon 20 days, while it was 85% with the non-bacterized control. Even-though the strain 111-3 offered protection till 20 DAC, it was not prolonged further and there were only 10% of total plants surviving in the treatment upon 30 days of challenge. After 30 DAC, it was observed that EXTN-1 treated plots had 35% plants without wilt disease while only 5% plants survived in the non-bacterized control plot.

It was interesting that EXTN-1 did not show any antagonistic properties against *R. solanacearum in vitro*. This would mean that the mechanisms of disease suppression by EXTN-1 are other than antibiosis. *B. vallismortis* strain EXTN-1 had been earlier reported to induce systemic resistance against various pathogens (Park et al., 2001). Ahn et al. (2002) reported that the major mechanisms by which EXTN-1 bring about disease suppression in crops is by the induction of systemic resistance to the host plant. This resistance mechanism has been proven to be effective against bacterial, fungal and viral pathogens of different crops (Ahn et al., 2002; Park et al., 2006a; 2006b).

Nakaho et al. (2000) reported those structural defence modifications in tomato in the primary and the secondary xylem tissues as a means of host defence mechanism can directly reduce the severity of wilt disease. They also reported that the cell wall modifications significantly hampered the movement of *R. solanacearum* within the vascular tissues. Also, Grimault et al. (1994) reported that the host defence modifications could slow down the rate of bacterial (*R. solanacearum*) spread in tomato. These finding with reference to *R. solanacearum*-tomato system could be taken into account for the disease suppression in tomato as EXTN-1 has been reported to induce defence structural modifications in host plants (Park and Kloepper, 2000; Park

et al., 2001). We also demonstrated the hypersensitive reaction (HR), oxidative burst, lignifications and cell structural defence modifications in host plants upon treatment with EXTN-1 (Park et al., 2001; Jeun et al., 2001; Ahn et al., 2001). We reported the activation of PR-1a and PDF 1.2 defence genes in transgenic tobacco and Arabidopsis by treatment of the plants with EXTN-1 (Park and Kloepper, 2000), which suggested a salicylic acid (SA) mediated and jasmonic acid (JA)-ethylene mediated systemic resistance pathways in the host plant with EXTN-1. Sarowar et al. (2005) showed that the pathogenesis related genes responsive to ethylene pathway in transgenic tomato enhanced its resistance to bacterial wilt.

The present study demonstrated the potential of EXTN-1 to suppress bacterial wilt in tomato. The strain could be effectively used for biocontrol of bacterial wilt in tomato. It warrants further investigations in the molecular level to understand the detailed mechanisms involved. As motility is a virulence factor for *R. solanacearum* (Tans-Kersten et al., 2001) and is obviously necessary for chemotaxis through soil to optimal infection sites on host roots, experiments need also to be set up to understand whether rhizobacteria mediated modifications in the host plant or rhizobacterial metabolites in the rhizospheric niche has any role in hampering the motility of the pathogen.

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