J. Appl. Biol. Chem. **50**(3), 136-143 (2007)

Article

Improvement of Biological Control against Bacterial Wilt by the Combination of Biocontrol Agents with Different Mechanisms of Action

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Received November 3, 2006; Accepted September 11, 2007

Despite the increased interests in biological control of soilborne diesease for environmental protection, biological control of bacterial wilt caused by *Ralstonia solanacearum* have not provided consistent or satisfying results. To enhance the control efficacy and reducing the inconsistency and variability, combinations of specific strains of microorganisms, each having a specific mechanism of control, were applied in this study. More than 30 microorganisms able to reduce the activity of pathogen by specific mechanism of action were identified and tested for their disease suppressive effects. After *in vitro* compatibility examinations, 21 individual strains and 15 combinations were tested in the greenhouse. Results indicated three-way combinations of different mode of control, TS3-7+A253-16+SKU78 and TS1-5+A100-1+SKU78, enhanced disease suppression by 70%, as compared to 30-50% reduction for their individual treatments. This work suggests that combining multiple traits antagonizing the pathogen improve efficacy of the biocontrol agents against *Ralstonia solanacearum*.

Key words: *bacterial wilt, biocontrol agent, biological control, mode of control,* Ralstonia solanacearum

Biological control of the plant pathogen is of a paramount importance due to the recent increase in the public concern for environment. However, biological control of *Ralstonia solanacearum* that causes wilting is still limited. Bacterial wilt is one of the most severe diseases of the economically important crops such as tomato, pepper, potato, and banana. *R. solanacearum*, a soil inhabitant, is transmitted to the plant roots via wounds or where the secondary roots emerge. The bacterium then colonizes the root cortex, invades xylem vessels and rapidly spreads throughout the vascular system, causing wilt and decay of the infected tissues [Kloepper *et al.*, 1980]. It is difficult to eradicate *R. solanacearum* due to its capability to survive in soil and water habitats for a considerable periods of time.

The mechanisms involved in the disease suppression by the beneficial rhizobacteria are diverse and include the bacterial production of metabolites, such as antibiotics, hydrogen cyanide, and lytic enzymes, which directly inhibit the pathogens, and competition for nutrients [Van Elsa *et al.*, 2000]. An alternative mechanism for the biological control by the rhizobacteria involves the induction of systemic resistance in plants [Van Loon *et al.*, 1998]. The induced resistance is defined as a state of the increased defensive capability developed by the plant through the activation of host defense system when appropriately stimulated by diverse agents including rhizobacteria. Once resistance is induced, it will afford non-specific protection against various pathogens and it is dependent on colonization of the root system by sufficient numbers of rhizobacteria [Hoffland *et al.*,1996].

Use of the beneficial rhizobacteria to suppress the plant disease appears to have a tremendous potential. However, biological control of soil-borne plant pathogens in the field has given inconsistent results due to the complexity of the biotic and abiotic interactions that play roles in biocontrol. Most approaches for biocontrol of plant diseases have used a single strain as an antagonist to a single pathogen. This may partially account for the reported inconsistent performance by biocontrol preparations, because a single biocontrol agent is not likely to be active in all soil environments where it was applied or against all races of pathogen that attack the host plant. Moreover, a significant genetic variation in *R. solanacearum* was reported [Hayward, 1991].

One possible approach to improve the biological control consistency may be the application of combinations of the biocontrol agents. By forming mixtures of biocontrol

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agents possessing different mechanisms of disease suppression, it could be assumed that at least one biocontrol mechanism will be functional under the conditions faced by biocontrol agents. It is also likely that most cases of naturally occurring biological control result from mixtures of antagonists, rather than from high populations of a single antagonist [Janisiewicz, 1996; Lemamceau and Alabouvette, 1991]. Compared with the usage of a single antagonist, application of a mixture of the antagonists might broaden the spectrum of the biocontrol activity and enhance the efficacy and reliability of control by allowing the combination of various mechanisms.

The objectives of this work were to verify the disease suppression by the biocontrol agents screened on the basis of their mode of action and to determine whether the improved efficacy could be achieved by applying combinations of biocontrol agents possessing different mechanisms against *R. solanacearum*, as compared to the use of a single microorganism.

Materials and Methods

Isolation of pathogen. An isolate of *R. solanacearum* was obtained from the infected tomato and pepper shoots by streaking the bacterial ooze onto the tryptic soy agar (TSA, Difco Laboratories, Detroit, MI). Creamy white virulent colonies of *R. solanacearum* were selected after 2 days incubation at 28°C on a tetrazolium chloride medium (TZC, Sigma Chemical Co, St. Louis, MO).

Isolation of rhizobacterial strains. Potential biocontrol strains were isolated from rhizosphere soil near the root surfaces of the tomato and pepper plants from bacterial wilt suppressive soil. After vigorous shaking of 1 g rhizosphere soil in 100 mL of the sterile distilled water for 15 min in a shaker, serial dilutions were made. One milliliter of each diluted solution was spread on TSA or King's B agar plates, and incubated at 28°C for 24 h. The potential biocontrol agents were selected according to their pathogen-inhibition zones on the artificial media, siderophore production and their root-colonizing abilities. The selected cultures were maintained at −80°C in a tryptic soy broth (TSB, Difco Laboratories, Detroit, MI) containing 20% glycerol for long-term storage.

***In vitro* antibiosis test.** Tests for the antibiotic activity of each strain against the pathogen were conducted using a paper disc method. A filter paper disk was impregnated with the culture filtrate of microorganisms and placed on a pathogen- seeded TSA plate. The plate was incubated for 48 h, and the sizes of inhibition zone were measured.

Siderophore production assay. The production of a siderophore was detected by the chromogenic assay on chrome azurol S (CAS) agar plates [Schwyn and Neilands, 1987]. CAS agar plates were prepared as follows; 60.5 mg CAS was dissolved in 50 mL distilled water and mixed with 10 mL iron(III) solution (1 mM $FeCl_3$, 10 mM HCl). Under continuous stirring, the solution was slowly mixed with 72.9 mg hexadecyltrimethylammonium bromide dissolved in 40 mL water. The resultant dark blue dye solution was autoclaved and mixed with an autoclaved mixture of 900 mL water, 19 g agar, 30.24 g of 1,4-piperazine diethane sulfonic acid (PIPES) and adjusted the pH to 6.8 using 1 N NaOH. A siderophore producing colony chelates iron from the medium, resulting in the color shift from blue to orange. After the droplets of an overnight culture were spotted on plates and incubated for 24 h at 28°C, the halo sizes were measured.

Studying rhizosphere colonization. Seeds bacterized with the test strains were allowed to germinate in the water agar plates. The root colonization ability is detected by the presence of a turbid zone in the agar plate around the roots and the root length was measured 5 days after germination at 28°C and compared with those of the non-bacterized seeds.

Seed bacterization. For seed treatment, bacteria were applied to tomato and pepper seeds according to the method of Ownley *et al.* Briefly, bacteria were cultured on TSA plates for 24 h and suspended in 0.02 M phosphate buffer, pH 7.0, followed by the addition of an equal volume of the 2% methylcellulose (Sigma, St. Louis, Mo.) solution. The seeds were coated with the bacteria by mixing 2.5 mL bacterial suspension with 5 g seeds. The coated seeds were dried overnight under a stream of sterile air at room temperature. Seed treatments with a single strain resulted in the population densities of 10^7 to 10^8 CFU per seed, and strain mixtures in which each strain was applied in equal amounts yielded 10^8 to 10^9 CFU per seed, which were verified by the dilution plating method before planting. The control was treated with 2% methylcellulose solution and no bacterium was detected by the dilution plating method.

Compatibility between the selected strains. *In vitro* compatibility tests were conducted on the selected biocontrol agents. Bacterial suspensions were made by suspending the cells cultured for 24 h at 28°C on the TSA plates in sterile water and the densities of bacterial suspensions were determined spectrophotometrically at 600 nm. The bacterial strains were adjusted to 10^7 CFU/mL and 100 μL of the suspension of target strain was spread over the agar surface and dried. Subsequently, a drop of each strain (10 μL) was spotted on the agar plates, approximately 1.5 cm from the edge. The spot- inoculated plates were incubated at 28°C for 24 h, and growth

inhibition zones of the target strain around the spot-inoculated strains were measured.

Greenhouse pot assays. The selected strains were evaluated under the greenhouse conditions for control of the tomato and pepper bacterial wilts. Bacterized and non-bacterized seeds of pepper and tomato were planted in 10-cm plastic pots containing soilless pro-mix, Baroker sangto. Each pot contained one plant. When plants were 30 days old, the bacterial strains were introduced individually and in combinations. Density of the individually applied biocontrol agents was 10^7 CFU/g of potting soil. When combinations of strains were used, each strain was introduced at a density of 10^7 CFU/g. Two days after the treatment with the biocontrol agents, the plants were challenged with the pathogen. *R. solanacearum* suspension (10^8 CFU/ mL) was made by suspending the cells cultured for 2 days at 28°C on TZC agar plates and 30 mL of the pathogen suspension was directly poured into the pots containing roots injured manually with a scalpel, thereby creating lesions and increased disease vulnerability. A water drench was used as the non-treated control. Treatments were arranged in a randomized complete block design with 10 replications. The experiment was conducted in triplicates. Two to 3 weeks after the pathogen challenge, the wilt disease was visually rated by assessing the percentage of leaf wilted. Disease severity ratings were based upon a scale in which 0 = healthy, 1 = slightly wilting (less than 25% wilted), 2 = moderate wilting (26-50% wilted), 3 = severe wilting (51-75% wilted) and 4 = 76-100% wilted or dead. Biocontrol efficacy was calculated using the following formula: Biocontrol efficacy = ([disease incidence of control-disease incidence of treatment group]/disease incidence of control) × 100.

Results

Selection of biocontrol agents based on mode of action. Potential biocontrol agents were selected according to the mechanisms involved in the biological control, such as antibiosis against pathogens, siderophore production and root colonizing abilities.

***In vitro* antibiosis.** Potential biocontrol agents were selected according to their pathogen-inhibition zones on the TSA media and the inhibitory effects were quantified by measuring the diameter of the inhibition zone. Upon examination of 300 isolates from the rhizospheres of healthy tomato and pepper plants growing in the suppressive soil, 17 colonies produced antibiotics against *R. solanacearum* (Fig. 1), among which 6 strains (A27-3, A48-2, A79-2, A100-1, A253-16 and A256-10 strains) exhibited the highest antibiotic activities (>3 mm diameter).

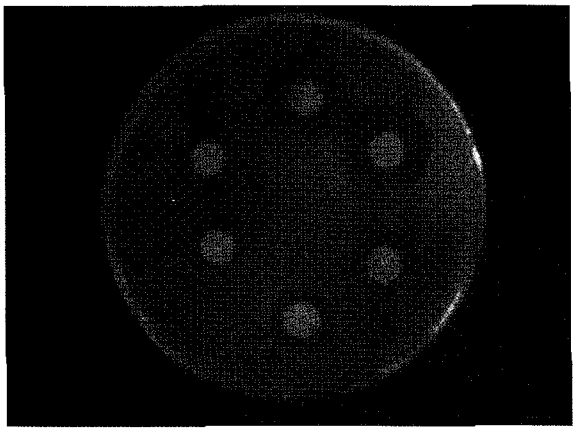

Fig. 1. *In vitro* inhibition of *Ralstonia solanacearum* on TSA medium by the selected microorganisms. Inhibition effects are evaluated by inhibition zone diameter.

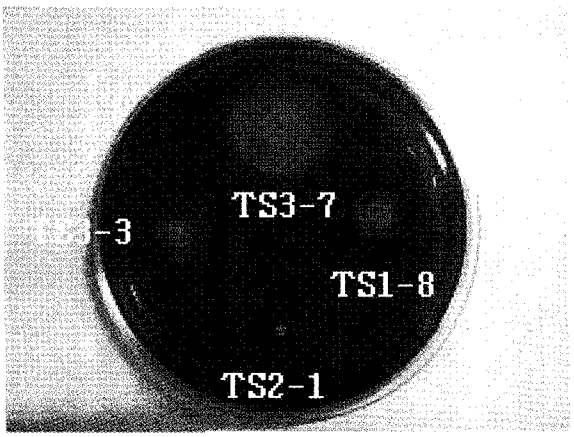

Fig. 2. Orange halo zone of siderophore producing strains on CAS blue agar.

Siderophore production. Production of a siderophore was suggested as a powerful mode of action in disease suppression solely based on the competition for iron with the pathogen [Backman *et al.*, 1997; Schippers *et al.*, 1987]. When the siderophore, a strong chelator, removes iron from the dye, its color turns from blue to yellow. Thirteen colonies produced halos on the CAS medium, 10 of which colonized on the root surface but showed no direct antibiosis (Fig. 2). In addition, three strains, TS1-2, TS3-7 and TP5-4 showed direct antibiosis against the pathogen *in vitro*.

Root colonizing competition. An important trait of the biocontrol agents is their ability to effectively colonize the rhizosphere and maintain a stable relationship with the surface of the plant roots. The root colonization is detected by the presence of a turbid zone around the roots in the agar plate. Eleven isolates produced the turbid zones in the agar plate around the roots and resulted in more than 10% enhancement of root growth (Table 1).

Table 1. Effects of the selected rhizobacteria strains on root elongation of pepper plant

Strains	SKU-7	SKU-65	SKU-78	SKU-132	SKU-134	SKU-155
Root elongation rate (%)	10.3	10.2	15.2	10.3	14.2	12.8
Strains	TS1-5	TS1-7	TS2-7	TS4-3	TS6-4	
Root elongation rate (%)	12.6	11.4	10.8	10.2	13.8	

Mean root length in millimeters was measured at 10 days after treatment. An equal number of plants were treated with sterile distilled water as control.
Root elongation rate = {(root length of treatment group-root length of control)/root length of control} ×100.

These strains were further tested both for *in vitro* antibiosis and their ability to control bacterial wilt disease of tomato and pepper under greenhouse conditions. The selected rhizobacteria were not *in vitro* antibiotic against the pathogen but suppress the disease symptoms in plants (*Vide infra*).

In the primary screening for potential biocontrol agents on the basis of control mechanisms, 300 isolates from the rhizospheres of healthy tomato and pepper plants growing in suppressive soil were tested, and 21 were selected. Six strains produced antibiotics against *R. solanacearum*, 9 produced siderophores and 6 colonized the root surfaces but showed no direct antibiosis.

Disease suppression by single microorganism. The microorganisms were selected on the basis of mechanisms of control in the primary screening and the efficacies of the selected microorganisms on the suppression of wilts were assessed through the greenhouse pot assay. In the control treatment, initial symptoms of wilting during the daytime and recovering at night occurred 3 days after the challenge and the wilt developed throughout the whole plant thereafter. The average biocontrol efficacies of 21 individual strains are listed in Table 2. Individual microorganism never exhibited any negative effects on the growth of plant, but some strains were found to be effective in increasing the plant growth (data not shown). From these microbial candidates, 12 strains having 3 different mechanisms of action were selected based on their performances as biocontrol agents; 3 strains (TS6-4, SKU78 and SKU134) having high root colonizing capacities, 5 strains (TS1-2, TS1-5, TS3-7, TS3-9, TP1-2 and TP5-4) producing siderophore, and 3 strains (A48-2, A100-1 and A253-16) with direct antibiosis. TS6-4, SKU78 and SKU134 strains, which have good root colonizing capacities, were not *in vitro* antibiotic against the pathogens, but significantly suppress disease symptoms in the plants. Siderophore-producing organisms, TS 3-7 and TS 1-2 showed antibiosis and also induced the highest suppressive effects.

Three modes of applications of these selected strains were tested: seed treatment, soil drench, and seed treatment + soil drench. Results presented here demonstrated that

Table 2. Suppression of bacterial wilt of tomato and pepper plants by the selected strains

Suppression mechanism	Strain	Biocontrol efficacy (%)[a]	
		pepper	tomato
Root colonization	TS1-7	35.0 (1.48)	28.3 (1.22)
	TS4-3	31.0 (1.29)	15.2 (1.23)
	TS6-4	37.5 (1.33)	30.3 (1.29)
	SKU65	32.5 (1.14)	25.9 (1.08)
	SKU78	47.5 (1.26)	34.8 (1.44)
	SKU134	44.5 (1.38)	32.8 (1.32)
Siderophore production	TS1-2	37.5 (1.17)	40.3 (1.23)
	TS1-3	30.1 (1.09)	15.2 (1.26)
	TS1-5	37.5 (1.85)	30.8 (1.35)
	TS3-7	48.2 (1.22)	31.3 (1.34)
	TS3-9	42.5 (1.32)	31.3 (1.25)
	TP1-2	45.3 (1.36)	20.8 (1.41)
	TP2-5	32.9 (1.21)	23.6 (1.23)
	TP3-4	41.1 (1.22)	15.9 (1.27)
	TP5-4	38.7 (1.42)	38.6 (1.32)
Antibiosis	A27-3	41.8 (1.22)	32.4 (1.42)
	A48-2	47.5 (1.33)	30.2 (1.21)
	A79-2	34.2 (1.25)	18.8 (1.41)
	A100-1	42.4 (1.23)	38.8 (1.45)
	A253-16	43.7 (1.25)	33.2 (1.22)
	A256-10	41.3 (1.31)	32.7 (1.37)

[a]Data represent means of 10 replications of each treatment. The experiment was repeated three times.
Biocontrol efficacy = ([disease incidence of control-disease incidence of treatment group]/disease incidence of control) × 100.
Standard errors are given in parentheses.

individual microorganism treatments induced significant disease suppression, compared to the non-treated control. In general, soil drench of bacterial suspension resulted in improved suppression than seed treatment. However, seed treatment followed by soil drench application appeared to be more effective than a single treatment, and resulted in a high level of reduction of the bacterial wilt disease; especially, SKU-78 and TS3-7 strains suppressed the disease symptoms up to 60% (Table 3).

Table 3. Suppression of bacterial wilt of tomato and pepper plants by the selected strains

Strains	Biocontrol efficacy (%)[a]					
	Seed treatment[b]		Soil application[c]		Seed + Soil treatment	
	Pepper	Tomato	Pepper	Tomato	Pepper	Tomato
TS1-5	17.6 (0.92)	10.3 (0.87)	37.5 (1.85)	30.8 (1.35)	42.3 (0.87)	40.5 (0.83)
TS6-4	23.5 (1.12)	15.4 (1.09)	37.5 (1.33)	30.3 (1.29)	40.1 (0.75)	39.7 (0.89)
SKU78	41.2 (1.03)	28.2 (1.06)	47.5 (1.26)	34.8 (1.44)	59.4 (0.65)	52.5 (0.88)
TS3-7	35.3 (1.35)	23.1 (1,03)	48.2 (1.22)	31.3 (1.34)	53.4 (0.68)	42.1 (0.45)
SKU134	41.2 (0.93)	15.4 (0.97)	44.5 (1.38)	32.8 (1.32)	50.2 (0.48)	34.7 (0.55)
A253-16	21.8 (1.01)	22.8 (1.12)	43.7 (1.25)	33.2 91.22)	47.5 (0.59)	43.2 (0.68)

[a]Data represent means of 10 replications of each treatment. The experiment was repeated three times. Disease severity was rated 10 days after inoculation with *R. solanacearum*. Biocontrol efficacy is based on comparisons to the nonbacterized but pathogen-challenged controls.

[b] Pepper and tomato seeds were coated with the selected bacterial cell suspension and planted.

[c] Plants were grown in potting soil and was drenched with bacterial cell suspension.

Biocontrol efficacy = ([disease incidence of control-disease incidence of treatment group]/disease incidence of control) × 100.

Table 4. Antagonism between the selected strains on the plate *in vitro*

Indicator strains	Test strains											
	TS6-4	TS1-2	TS1-5	TS3-7	TS3-9	TP1-2	TP5-4	A48-2	A100-1	A253-16	SKU78	SKU134
TS6-4	-	-	+	-	+	-	-	-	-	-	-	-
TS1-2	-	-	-	+	-	-	-	-	-	-	-	+
TS1-5	-	+	-	-	+	-	-	-	-	-	-	+
TS3-7	-	-	-	-	-	+	+	-	-	-	-	-
TS3-9	-	-	-	-	-	+	-	-	+	-	-	+
TP1-2	-	-	-	+	+	-	-	+	-	-	-	+
TP5-4	-	-	+	-	-	-	-	-	-	+	+	-
A48-2	-	-	-	-	-	-	-	-	-	-	-	-
A100-1	-	-	-	-	+	+	-	-	-	-	-	-
A253-16	-	-	-	-	-	-	-	-	-	-	-	+
SKU78	-	-	-	-	-	-	-	-	-	-	-	-
SKU134	-	+	-	+	-	-	-	-	-	+	-	-

Microbial antagonisms were tested on nutrient agar plates.
+; antagonistic activity exhibited, -; no antagonistic activity.

Compatibility between the selected strains. Interactions between the introduced strains can positively influence the growth and/or root colonization, thereby enhancing the disease suppression. However, incompatibility of the co-inoculants can also arise. For instance, competition for substrates or root colonization among the introduced strains may negatively influence the disease control. Therefore, as an important prerequisite for a successful development of the strain mixtures, compatibility of the co-inoculated microorganisms was evaluated (Table 4). Microbial candidates with different mechanisms of control and no antagonistic activities against each other were combined and examined for their disease suppressive effects.

Disease suppression by combination of biocontrol agents with different mode of actions. One possible approach to improve the biological control is the application of mixtures of biocontrol agents having different mechanisms. Combining the microorganisms could provide an insurance that at least one biocontrol mechanism will be functional under the environmental conditions faced by the biocontrol agents. Moreover, combination of the biocontrol strains could have a potential to suppress multiple plant diseases. By combining specific strains of microorganisms, multiple traits antagonizing the pathogen can be combined and this may give better suppression

Table 5. Suppressive effects of the strain mixtures on the bacterial wilt disease in potted tomato and pepper plants.

Combinations	Suppressive effects (%)[a]	
	Pepper	Tomato
① TS1-2 +A48-2 + SKU78	52 (1.02)	45 (1.21)
② TS1-2 +A100-1 + SKU78	57 (0.98)	48 (1.09)
③ TS1-2 + A253-16 + SKU78	52.5 (0.97)	42.5 (0.88)
④ TS1-5 +A48-2 + SKU78	54.5 (1.02)	43.5 (1.21)
⑤ TS1-5 +A100-1 + SKU78	65 (1.05)	70 (1.08)
⑥ TS1-5 + A253-16 +SKU78	56 (1.12)	42.5 (1.23)
⑦ TS3-7 + A48-2 + SKU78	57.5 (0.98)	43.5 (1.01)
⑧ TS3-7 +A100-1 + SKU78	53 (0.68)	42.5 (1.19)
⑨ TS3-7 + A253-16 + SKU78	75 (0.78)	70 (0.89)
⑩ TS3-9 + A48-2 + SKU78	55 (1.24)	44 (0.78)
⑪ TS3-9 + A253-16 + SKU78	50.5 (0.95)	47.5 (0.88)
⑫ TP1-2 + A48-2 + SKU78	57.5 (1.23)	47.5 (1.01)
⑬ TP1-2 + A253-16 + SKU78	55 (0.72)	46 (1.12)
⑭ TP5-4 + A48-2 + SKU134	50 (0.88)	45 (1.13)
⑮ TP5-4 + A100-1 + SKU134	55 (1.23)	47.5 (0.79)

[a]Data represent means of 10 replications of each treatment. The experiment was repeated three times. Disease severity was rated 10 days after inoculation with *R. solanacearum*. Biocontrol efficacy is based on comparisons to the nonbacterized but pathogen-challenged controls.
Biocontrol efficacy = ([disease incidence of control-disease incidence of treatment group]/disease incidence of control) × 100.
Standard errors are given in parentheses.

than a single treatment with the individual strains. Disease suppressions by fifteen combinations of compatible microorganisms, each of which has a characteristic mechanism of action, were investigated through the potting soil bioassay (Table 5). These mixtures exhibited three different biocontrol mechanisms, including production of antibiotics, the competitions for ferric ion and competition for infection sites. Generally, mixtures exhibited higher disease suppression than those used individually. In particular, the combinations of TS1-5+A100-1+SKU78 and TS3-7+A253-16+SKU78 resulted in significantly enhanced disease suppression when applied as soil drench treatments.

Discussion

The results indicated that several individual strains and several mixtures provided significant disease suppression against the bacterial wilt under the greenhouse condition. Active and long-term colonization of the root surface of the plant is generally considered prerequisites for successful suppression of the soilborne disease caused by the rhizobacteria directly through the production of antimicrobial substances or competition for space, nutrients and ecological niches, or indirectly through the induction of a systemic resistance. Due to the absence of detectable *in vitro* antagonism against the pathogen, it was considered that observed disease suppressive effects of these root-colonizing strains might be due to induced systemic resistance (ISR) [Handelsman and Stabb, 1996]. ISR is a state of the enhanced defensive capacity developed by a plant when appropriately stimulated and is regarded as the mode of action of the disease suppression by the non-pathogenic rhizosphere bacteria. Non-specificity is an important advantage of ISR as compared to the classical biological control, in which the antagonist selected is generally active against only one or a few pathogens. Besides direct antibiosis and induction of resistance of the host plant by the biocontrol agents, competition for iron by siderophore-producing strains could not be excluded from mode of action that can suppress the bacterial wilt. In addition, siderophores have been known to play a role in the induction of resistance of the host plant against different diseases [Leeman *et al.*, 1996].

The *in vitro* antibiosis study showed that SKU 78 and SKU 134 did not function as antibiotic against *R. solanacearum*, suggesting but not proving that the mechanism of control may be induced systemic resistance. TS3-7 and TS1-2 exhibited at least two control mechanisms, antibiosis and iron competition. The three-way combinations, TS3-7+A253-16+SKU78 and TS1-5+A100-1+SKU78, each having a distinct control mechanism, showed the

improvements in control efficacy compared with their separate application. Since root colonization ability, antibiosis and competition for ferric ion by siderophore production are separate mechanisms of disease suppression by rhizobacteria, the combinations of these modes of action may well lead to improved efficacy of biological control. The antibiosis would first weaken or even partly kill the population of the pathogen and subsequently, this weakened population of the pathogen would be confronted with a plant that is in a state of enhanced defensive capacity, thus resulting in much less disease development. Effective competition for iron in the rhizosphere, also contribute to disease suppression.

In response to environmental and health concerns about pesticides, it seems inevitable that fewer pesticides will be used in the future and that greater reliance will be placed on biological technologies including the use of microorganisms as antagonists. However, microorganisms as biological control agents typically have a relatively narrow spectrum of activity compared with synthetic pesticides. Due to the complexity of the biotic and abiotic interactions that play a role in biological control, the results often exhibit inconsistent performance in practical agriculture, resulting in limited commercial use of biocontrol approaches for suppression of plant pathogens [Budge and Whipps, 2001; Kloepper *et al.*, 1999]. Apparently, one possible approach to improve biocontrol under fluctuating environmental conditions may be the application of combinations of biocontrol agents, particularly if they exhibit different or complementary modes of action. By combining microorganisms, multiple disease suppressive traits can be combined and at least one biocontrol mechanism may be functional under the conditions faced by biocontrol microorganisms and resulted in reduced variability of biocontrol effects. It was similarly demonstrated that combining proteolytic enzyme and antibiotics producing bacteria could improve biocontrol of *Pythium*-mediated damping-off of sugar beet [Dunne *et al*, 1998]. In most of the investigations conducted so far, the effects of individual biocontrol strain or strain combinations were assessed by these agents against a single isolate of the target pathogen. This might be another reason why selected biocontrol agents fail to be commercially successful, because often there is no consideration of the genetic diversity of the target pathogen. Little emphasis has been placed on identifying the potential effects of pathogen diversity on the efficacy of biocontrol agents. The significant genotypic variations in *R. solanacearum* have been reported among geographic regions and even within a given field. Therefore, use of several biocontrol agents with several mechanisms of action can be a novel approach to biological control against *R. solanacearum*. The current study have shown that three-way combinations of a particular strain, TS1-5+A100-1+SKU78 and TS3-7+A253-16+SKU78, gave a greater protection, compared to single strain treatments in pot assay. Further work is needed to prove whether these mixtures induced a higher protection level under field conditions.

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