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Control of Bacterial Wilt Disease Caused by *Ralstonia solanacearum* in Ginger and Postharvest Treatment by Antagonistic Microorganisms

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Abstract: Seventy-eight bacterial isolates and two commercially available microorganisms were evaluated for control of *Ralstonia solanacearum* race1 biovar 4, a bacterium wilt disease pathogen of ginger in Thailand. Two bacteria, *Bacillus subtilis* K1 and *Pseudomonas fluorescence* PS12 and the commercially available fungus *Trichoderma harzianum* AP-001 (Trisan™) provided the best disease control. In soil artificially infested with the pathogen and incubated in the laboratory, the three microorganisms reduced *R. solanacearum* population density after 9 days. In two greenhouse experiments, ginger plants were inoculated with *R. solanacearum* and then transplanted into soil containing powder formulations of *B. subtilis* K1, *P. fluorescence*, *T. harzianum* AP-001, or their combinations; the formulations were added at one time just before transplanting in the first experiment or in a split applications in the second experiment. The combination of *P. fluorescence* PS12 and *B. subtilis* K1 in a 1:2 ratio provided the highest level of control in both experiments; however, application once before transplanting provided longer control than the split application. Post harvest treatments dipped with the bacterial combination at $5x10^6$ cfu/mL showed better control than the commercial *T. harzianum* AP-001 at the same concentration after dipped in a 1%w/v NaOCl in a long term protection from bacterial rot.

Key words: *Bacillus subtilis* · Biological control · Ginger · *Pseudomonas fluorescence* · *Ralstonia solanacearum* · *Trichoderma harzianum* · Post harvest treatments

INTRODUCTION

The bacterium *Ralstonia solanacearum* (Smith), which was formerly known as *Pseudomonas solanacearum* EF Smith and *Burkholderia solanacearum* [1], causes bacterial wilt of ginger and other crops. Bacterial wilt is one of the most important and widespread diseases of plants in the family Zingiberaceae and is the most important disease of ginger in Asian planting countries [2]. The bacterium infects plants at the roots or lower stem. In addition, ginger rhizome contaminated by the bacterium resulted in rhizome rot after harvested. Therefore, the exporting enterprises in Thailand have to use some antibiotics or chemical fungicides for post harvest treatment in order to inhibit the growth of the bacterium. Unfortunately, the allowable levels of maximum residue limits of antibiotics and/or synthetic chemical fungicides are so low that these products could no longer be used. Therefore, microbial fungicides become an attractive alternation for post harvest treatment program.

The pathogen was divided into five races [3- 5]. Besides these races, four biovars of *R. solanacearum* have been characterized based on their ability to utilize and/or oxidize three hexoses (mannitol, dulcitol and sorbitol) and three disaccharides (lactose, maltose and cellobiose), [6,7].

Biological control of root diseases by antagonistic bacteria has been attributed to several mechanisms, including the production of a variety of antimicrobial compounds. For example, some *Bacillus subtilis* strains produce the iron chelating agent 2,3-dihydroxybenzoylglycine (2,3-DHBG), [8,9] and *Pseudomonas fluorescens* produces phenolate/ catecholate or hydroxamate compounds [10]. Similarly, antimicrobial metabolites such as iturin [11,12] are produced by *B. subtilis* and 2,4-diacetylphloroglucinol (DAPG) is produced by *P. fluorescens* [13 - 15]. Suppression of root diseases by antagonistic fungi, such as *Trichoderma harzianum*, results from competition and colonization and the production of antibiotic metabolites such as dermadine [16 - 19]. Only a few papers have

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reported on the biological control of *R. solanacearum* in ginger or other crops. Vilasini [20] studied *Trichoderma harzianum* for control of *R. solanacearum* in ginger. Ciampi *et al.* [21] described an alginate bead formulation of *Bacillus subtilis* A47 and *Pseudomonas fluorescense* BC8 for controlling *R. solanacearum* in potatoes and tomatoes. Thongwai and Kunopakarn [22] reported on growth inhibition of *R. solanacearum* PT1J in Siam Tulip or Patumma (*Curcuma alismatifolia* Gagnep.) by some antagonistic bacteria.

In our previous work [23], we reported on the efficacies of the antagonistic bacterium *B. subtilis* AP-01 in combination with the antagonistic fungus *T. harzianum* AP-001 for control of bacterial wilt in tobacco caused by *R. solanacearum* race 1 biovar 3 [24]. After describing an *in vitro* screen for inhibition of *R. solanacearum* by 78 new isolates of soil bacteria, the present paper reports on the control of bacterial wilt of ginger by *Bacillus subtilis* K1, *Pseudomonas fluorescence* PS12 and *T. harzianum* AP-001. The two bacteria were identified in the *in vitro* screen whereas the fungus is available in a commercial formulation. Post harvest treatments with these bacteria and a commercial fungus were also reported.

MATERIALS AND METHODS

Isolation of the Pathogen: *Ralstonia solanacearum* was isolated from diseased rhizomes of *Zingiber officinale* obtained from the following four commercial plantations that produce edible ginger: Amphoe Nam Nao in Petchabun Province (16°46′6″N, 101°40′18″E); Amphoe Phu Ruea in Loei Province (17°27′18″N, 101°21′48″E); Amphoe Phu Khiao in Chaiyaphum Province (16°22′35″N, 102°7′43″E); and Amphoe Bang Saphan in Prachuap Khiri Khan Province (11°12′54″N, 99°30′42″E). From each plantation, segments of diseased stems, 2-3 cm above soil level, were washed with tap water, surface disinfested in 70% ethanol for 2-3 min and washed with sterile-distilled water. The stem segments (one to three per plantation) were then placed in 5 ml of sterile-distilled water for 10-15 min. The surface-disinfested segments were cut, releasing the bacterial ooze from the vascular system into the suspension. The suspension was then streaked onto PSA (Wakimoto's potato semi-synthetic agar) and triphenyl tetrazolium chloride (TTC) medium [25] and incubated at 30°C for 2-3 days. Single bacterial colonies were transferred to fresh PSA and TTC plates. Creamy white colonies with pink-red center occurred on TTC medium. The creamy white colonies were transferred to 5 mL of sterile-distilled water in 16-cm^3 screw-top tubes and represented stock suspensions.

Biochemical Tests for Biovar Classification: The method of Thammakijjawat and Kositchareonkul [26] was employed for biovar determination.

Virulence of the Four R. Solanacearum Isolates: Ten, 60-day-old ginger plants were inoculated with each of the four *R. solanacearum* isolates. The stem and root of each plant was injured by the techniques of Somodi *et al.* [27] and were inoculated with *R. solanacearum*. A micropipette was used to add 0.5 mL of a suspension containing $2.7x10^7$ cfu mL^{-1} to each wound. Negative control plants were wounded in the same manner but inoculated with sterile-distilled water.

Selection of Antagonistic Microorganisms: Soil samples (120) from the rhizoplanes of healthy plants were obtained from the following crop plantations: ginger, tomato, eggplant and potato. Eighty organic materials (compost, green manure and rice hulls) were also collected from the four provinces mention earlier. For each of the 500-g samples of soil or organic materials, 10 g was put into a 250-mL sterile bottle containing 90 mL of sterile-distilled water; the bottle was shaken at 150 rpm for 1 hr at room temperature. The suspensions were diluted (1:10) and 1 mL of each dilution was added to plates containing King-B medium. The plates were kept at 30°C for 3-5 days before a single bacterial colony was collected from each plate and transferred to a PSA plate. If growth on the PSA plate indicated a pure colony, two loops of each colony were transferred to a tube containing 5 ml of sterile-distilled water. These tubes served as stock cultures for the 78 isolated bacteria.

Two commercial wettable powder products (Larminar™ and Trisan™) were obtained from Appliedchem Co., Ltd. (Thailand). Larminar™ contained $1x10^9$ cfu of *B. subtilis* AP-01 g^{-1} and Trisan™ contained $2x10^8$ cfu of *T. harzianum* AP-001 g^{-1}.

***in vitro* Screening for *R. solanacearum* Antagonists:** A paper-disk diffusion method [28] was used to screen the 78 isolates for activity against *R. solanacearum*. After NGA (nutrient glucose agar) medium was poured into Petri dishes (5 ml per dish) and allowed to cool and

form a basal layer, 0.5 ml of the selected *R. solanacearum* at $2.7x10^7$ cfu ml^{-1} was mixed with 5 ml of melted warm NGA and poured on the surface of the basal layer.

Suspensions of every antagonistic bacterium were prepared at about $1.0x10^8$ cfu/ml. Four disks (0.5 cm diameter) of autoclaved Whatman # 1 filter paper were dipped into each bacterial suspension and placed on the surface of an NGA plate with *R. solanacearum*. Disks that were dipped in sterile-distilled water served as a control. Each isolate was represented by three replicate plates. After 48 hr at 33°C, clear zones that formed around the filter paper disks were measured.

Preparation of Powder Formulations: The two isolates (a *Bacillus* sp., isolate K1 and a fluorescent pseudomonad, isolate Ps12) that produced the largest clear zones in the paper-disk diffusion test were each formulated as a wettable powder followed the method of Maketon *et al.* [29] to give $1x10^9$ cfu g^{-1} of formulation. The formulations were kept at 4°C.

Effects of Antagonists on *Ralstonia solanacearum* Numbers in Soil Incubated in the Laboratory: Soil (2 kg; 2.0% organic matter, 28% sand, 55% silt and 15% clay) from a ginger planting area in Petchaboon Province was autoclaved at 121°C for 20 min. After the soil had cooled, the soil moisture content was adjusted to 50% (g of water 100 g^{-1} g dry soil x 100) by addition of distilled water. A suspension of the most virulent *R. solanacearum* isolate was mixed with the autoclaved soil to yield $4.4x10^7$ cfu/g of soil. The infested soil was placed in 250-ml Erlenmeyer flasks (50 g of soil $flask^{-1}$) and the flasks were sealed with a cotton plug and incubated overnight at 30°C in the dark. Antagonistic microorganisms (K1, Ps12, or Trisan™), either in the suspension or powder form and either single or combined, were added to each flask (Table 1). The positive control consisted of flasks with *R. solanacearum* but without antagonists and the negative control consisted of flasks without *R. solanacearum* or antagonists. Each treatment was represented by three replicate flasks. After 0, 3, 5, 7 and 9 days at 25°C, soil dilution plating was conducted by removing 5 g of soil from the flask, suspending the soil in 50 ml of sterile-distilled water and spreading a 1-ml aliquot on TTC medium; three additional serial dilutions (10 fold) were prepared in the same manner. The concentration of *R. solanacearum* cells was calculated as colony forming units g^{-1} of soil.

Effects of Antagonists on Bacterial Wilt of Ginger in the Greenhouse: Soil was collected from Petchaboon Province (see previous section) and autoclaved at 121°C for 10 min. A suspension of *R. solanacearum* was then mixed into the soil to give $2.7x10^6$ cfu g^{-1} of soil. The infested soil was added to plastic pots (500 g of soil pot^{-1}). In the first greenhouse experiment, 30 g of antagonists was added to the 500 g of soil before it was added to pots (Table 2). Three days after the soil was added to pots, one 60-day-old ginger plant was transplanted into each pot. The plants had been inoculated with *R. solanacearum* just before transplanting as described earlier in the section concerning the virulence of *R. solanacearum* isolates. Pots with *R. solanacearum* but without antagonists or without *R. solanacearum* or antagonists served as controls (Table 2). Each treatment was represented by six replicate pots.

The second experiment was identical to the first except that a split application was used, in that 15 g of antagonist formulation was mixed into the soil in each pot before transplanting and another 15 g was added 7 days after transplanting.

The greenhouse was maintained at 28-30°C, 75-80% relative humidity and with ambient light. Pots were watered as needed but were not fertilized. After 0, 3, 7 and 9 days, the ginger plants were assessed for disease according to a disease index rating system of 0 to 5 [30]: 0 = no disease symptoms; 1 = leaves have begun to wilt but are still green; 2 = leaves have begun to curl and turn yellow at the tip; 3 = the lower leaves have curled and turned yellow while the 2-3 upper leaves are still green; 4 = all leaves have curled and turned yellow; and 5 = the stem has wilted and collapsed and the rhizome has begun to rot.

$$\text{The average disease development index} = \text{Sum (each category index x number of plants in that category)/total plants}$$

$$\text{Control efficiency percentage} = \frac{(5\text{-Average disease development index})}{5} \times 100$$

Effects of Antagonists on Post Harvest Treatment: A semi-commercial condition was conducted [31]. Twenty kilograms ginger rhizomes (12 months old) were harvested from Petchaboon Province packed in four card boxes and transferred to the laboratory. They were washed with tap water thoroughly and then cut with a sterilized knife to

Table 1: Treatments used to test the effects of *Bacillus subtilis* K1, *Pseudomonas fluorescence* PS12 and *Trichoderma harzianum* AP-001 (Trisan™) on numbers of *Ralstonia solanacearum* in soil

Treatment	Quantity applied per 50 g soil
1. K1 cell suspension (10^9 cfu/ml)	5 ml
2. PS12 cell suspension (10^9 cfu/mL)	5 ml
3. K1 powder (10^9 cfu/g)	3 g
4. PS12 powder (10^9 cfu/g)	3 g
5. Trisan™ powder (10^8 cfu/g)	3 g
6. K1 powder + Trisan™ ratio 1:1	3 g
7. PS12 powder + Trisan™ ratio 1:1	3 g
8. K1 powder + PS12 powder ratio 1:1	3 g
9. K1 powder+PS12 powder+Trisan™ ratio 1:1:1	3 g
10. Positive control	No antagonist added
11. Negative control	No pathogen or antagonist added

Table 2: Treatments used to test the effects of *Bacillus subtilis* K1, *Pseudomonas fluorescence* PS12 and *Trichoderma harzianum* AP-001 (Trisan™) on bacterial wilt of ginger plants in the greenhouse

Treatment	Quantity applied per 500 g soil
1. K1 powder	30 g
2. PS12 powder	30 g
3. Trisan™ powder	30 g
4. K1 + Trisan™ ratio 1:1	30 g
5. K1 + Trisan™ ratio 1:2	30 g
6. K1 + Trisan™ ratio 2:1	30 g
7. PS12 + K1 ratio 1:1	30 g
8. PS12 + K1 ratio 1:2	30 g
9. PS12 + K1 ratio 2:1	30 g
10. PS12 + Trisan™ ratio 1:1	30 g
11. PS12 + Trisan™ ratio 1:2	30 g
12. PS12 + Trisan™ ratio 2:1	30 g
13. Positive control	No antagonist added
14. Negative control	No pathogen or antagonist added

make wounds. These rhizomes were randomly divided into five groups, four kilograms for each group. The first group was dipped in distilled water for 10 min, the second group was dipped in a 1%w/v NaOCl solution for 1 min, the third group was dipped in a 1%w/v NaOCl solution for 10 min, the fourth group was dipped in a 1%w/v NaOCl solution for 5 min, drained and continue dipped in the best bacterial combination suspension ($5x10^6$ cfu/ml) from greenhouse tested for 5 min and the last group was dipped in a 1%w/v NaOCl solution for 5 min, drained and continue dipped in Trisan™ suspension ($5x10^6$ cfu/ml) for 5 min. All were air dried overnight under room temperature (28±2°C). One kilogram of treated ginger rhizomes was packed into a card box; four card boxes were prepared as replications for each treatment. They were stored at 13°C and 65% relative humidity for 12 weeks and were brought out for visually observed and recorded the Disease index and Decay percentage (DI: 1-10; 1 = 100% healthy – 10 = 100% rotten).

Statistical Analysis: A completely randomized design was used for all experiments and data were subjected to an analysis of variance using SAS version 9.1.3.

RESULTS

Isolation of the Pathogen, Biochemical Tests for Biovar Classification, Virulence of the Four *R. solanacearum* Isolates and Selection of Antagonistic Microorganisms: One isolate of *Ralstonia solanacearum* was obtained from each of the four ginger plantations/provinces. Because all four isolates could oxidize hexose alcohol but not disaccharides, they were classified as biovar 4. These results were consistent with those of the Thai Department of Agriculture, who identified *R. solanacearum* in Thailand ginger as race 1 biovar 4 based on PCR [26].

Of the four isolates of *R. solanacearum*, the one from Petchaboon Province was the most virulent. It caused the wilting and collapse of all stems within 20 days after inoculation, while symptom development was slower with the other three isolates. The isolate from Petchaboon Province was used for all other tests.

***in vitro* Screening for *R. solanacearum* Antagonists:** Seventy-eight bacteria were isolated from soils, compost, green manure and rice hulls. These included six fluorescent pseudomonads and 72 *Bacillus* spp., which were preliminarily identified by using a bacillus card (Biomerieux sa, France). In the paper-disk diffusion test, the two best isolates (the two isolates that produced the largest clearing zone) were *Bacillus subtilis* (K1) and *Pseudomonas fluorescence* (Ps12) (Table 3). A commercial isolate, *B. subtilis* AP-01 (Larminar™), did not produce a clearing zone (data not shown).

Effects of Antagonists on *R. solanacearum* Numbers in Soil Incubated in the Laboratory: At the start of this experiment (day 0), the population density of *R. solanacearum* in soil was similar for all treatments other than the negative control ($F = 1063.27$, $df = 10$, $p < 0.05$) (Fig. 1a). At day 3, the population of *R. solanacearum* had decreased in treatment 5 (Trisan™ powder), treatment 6 (K1 + Trisan™ 1:1) and

Table 3: Paper-disc diffusion tests for antagonistic bacteria against *Ralstonia solanacearum*

Isolate	Code	Source	Antibiosis (clear zone)
1	TT1	Compost	+
2	TT2	”	+
3	TT3	”	-
4	TT4	”	-
5	BS0130651	green manure	++
6	BS0130652	”	+
7	BS0130653	”	+
8	BS0130654	”	+
9	BS0130655	”	++
10	BC1	Soil from ginger rhizosphere, Roei Province	+
11	BC7	”	+
12	BC12	”	+
13	BC17	”	+
14	BC22	”	+
15	BC29	”	+
16	BC35	”	+
17	BC39	”	+
18	BC42	”	+
19	BC45	”	+
20	AP01	From Appliedchem (Thailand) Co. Ltd.	++
21	K1	Soil from ginger rhizosphere, Petchaboon Province	+++
22	AP04	”	++
23	MK007	”	++
24	PS3	”	++
25	PS6	”	++
26	PS12	”	+++
27	PS15	”	++
28	PS18	”	++
29	PS20	”	++
30	AP08	”	+
31	BT-TR	rice hull	-
32	BT-DP	”	-
33	BT-DF	”	-
34	BT-BP	”	-
35	B-260505	”	-
36	F-260505	”	+
37	BS	”	+
38	PDA0501.1	compost	+
39	PDB0501.4	”	+
40	PDC0502.1	compost	+
41	PDD0502.3	”	+
42	PDF0501.6	”	+
43	PDG0501.7	”	+
44	PDU0501.1	compost	+
45	PDU0501.2	”	+
46	BS01	green manure	++
47	BS10	”	++
48	BC	”	-
49	BA	”	-
50	BC07	”	-

Table 3: Continued

51	CD01	"	+
52	CD02	"	+
53	CD03	"	+
54	BCA1	Soil from ginger rhizosphere, Chiyaphoom Province	++
55	BCA3	"	++
56	BCA4	"	+
57	BWD1	"	++
58	BWD2	"	+
59	BWD3	"	+
60	DDS1	Soil from ginger rhizosphere, Chiyaphoom Province	++
61	DDS2	"	+
62	DDS3	"	+
63	CF 1005-1	Soil from ginger rhizosphere, Prachuebkerekan Province	+
64	CF1005-2	"	++
65	CF 1005-3	"	+
66	CF 1005-4	"	+
67	CF 1006-1	"	+
68	CF 1006-2	"	+
69	CF 1006-3	"	+
70	CF 1006-4	"	+
71	CF 7015-1	"	++
72	CF 7015-2	"	+
73	CF 7015-3	"	+
74	CF 7015-4	"	++
75	AG 01	compost	+
76	AG 02	"	-
77	AG 03	"	-
78	AG 04	"	+
79	AG 06	"	+

Remark: - means no clear zone exhibition + means clear zone diameter = 0.5 cm
++ means clear zone diameter = 1.0 cm +++ means clear zone diameter = 1.5 cm

treatment 9 (K1 + PS12 + Trisan™ 1:1:1) ($F = 2090.50$, $df = 10$, $p < 0.05$) (Fig. 1b); similar treatment effects occurred at day 5 ($F = 4758.43$, $df = 10$, $p < 0.05$) and 7 ($F = 3827.94$, $df = 10$, $p < 0.05$), except for treatment 9 (Fig. 1c and d). At day 9, the population density of *R. solanacearum* had decreased in most treatments except in the positive control, which indicated that *R. solanacearum* could persist in the absence of antagonists ($F = 3903.96$, $df = 10$, $p < 0.05$) (Fig. 1e). Most antagonist treatments showed good potential for controlling *R. solanacearum* and K1 powder, PS12 powder, Trisan™ and their combinations were chosen for further greenhouse testing. However, the combination of K1 + PS12 + Trisan™ 1:1:1 powder was not used in the greenhouse experiments because the combination of the three antagonists did not perform better than the treatments with a one or two of the antagonists.

Effects of Antagonists on Bacterial Wilt of Ginger in the Greenhouse: At day 7 in the first greenhouse experiment, the best control of bacterial wilt was obtained with treatment 8 (the mixture of Ps12 and K1 at 1:2), followed by treatment 12 (Ps12 + Trisan™ at 2:1) and treatment 2 (Ps12) ($F = 52.18$, $df = 13$, $p < 0.05$) (Fig. 2a). At day 15, the combination of Ps12 + K1 at 1:2 still provided the best control while control by Ps12 + Trisan™ at 2:1 had substantially decreased ($F = 39.72$, $df = 13$, $p < 0.05$) (Fig. 2b). At day 20, Ps12 + K1 at 1:2 still provided > 70% control while the other treatments provided < 50% control ($F = 990.68$, $df = 13$, $p < 0.05$) (Fig. 2c).

At day 7 of the second greenhouse experiment, the best control of bacterial wilt was obtained with treatment 8 (Ps12 + K1 at 1:2), followed by treatment 12 (Ps12 + Trisan™ at 2:1) and treatment 9 (Ps12 + K1 at 2:1) ($F = 99.14$, $df = 13$, $p < 0.05$) (Fig. 3a). At day 10, control

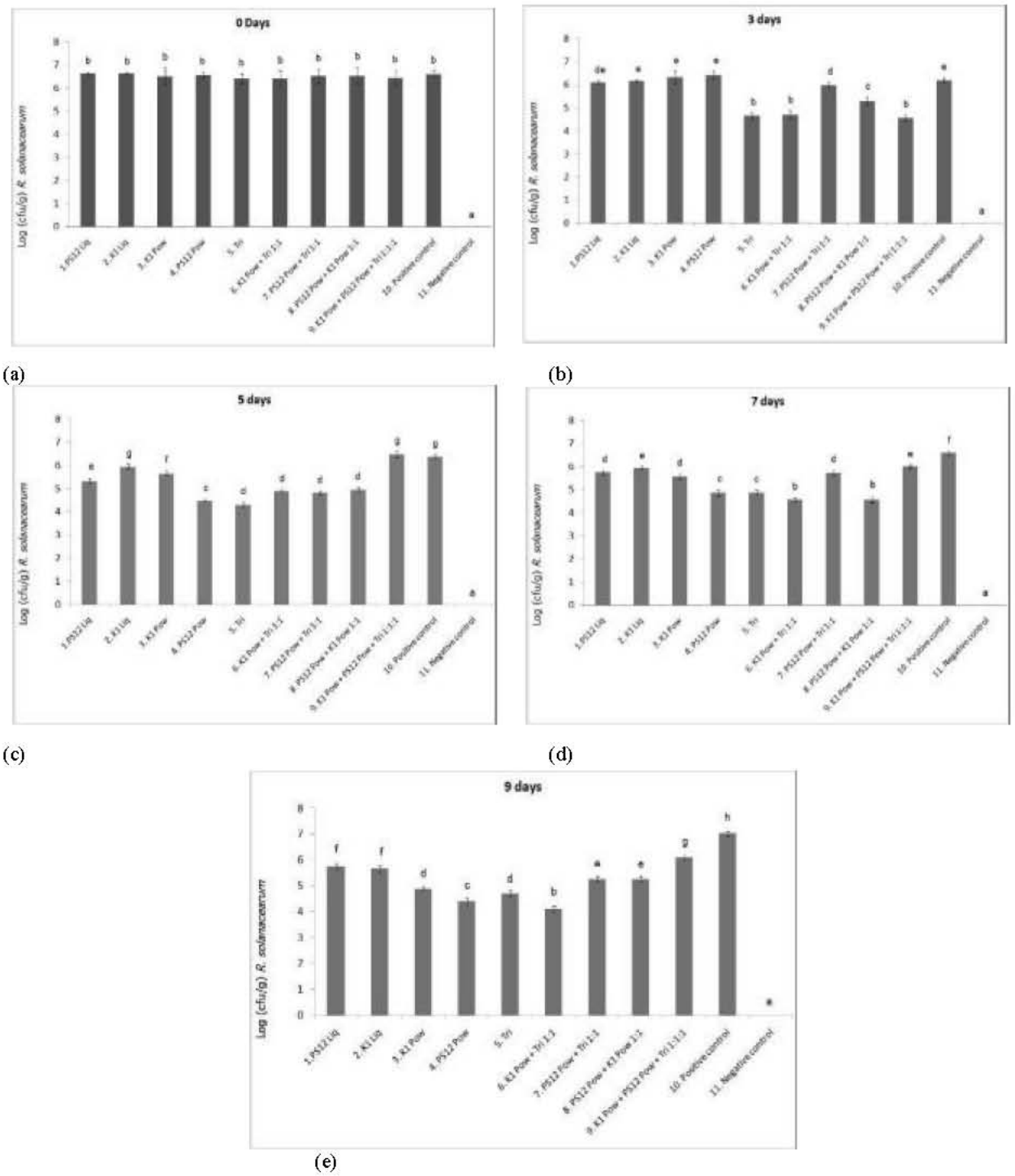
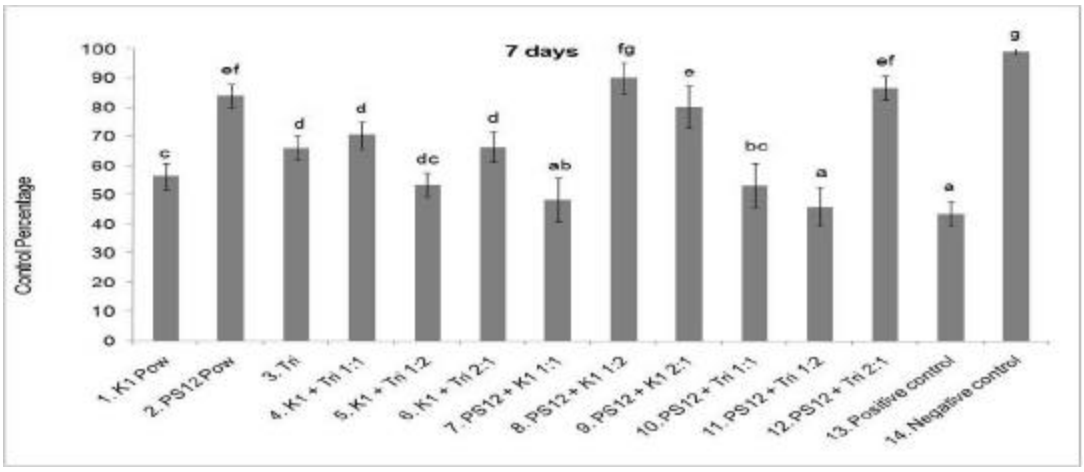
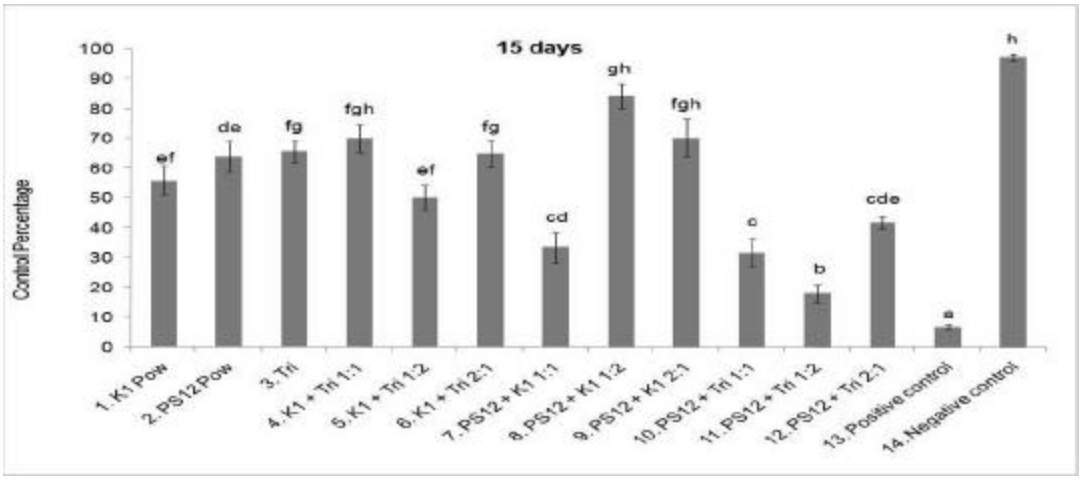


Fig. 1: Effects of antagonists on *Ralstonia solanacearum* numbers in soil incubated in the laboratory (a. at day 0 - b. at day 3 - c. at day 5 - d. at day 7 - e. at day 9)

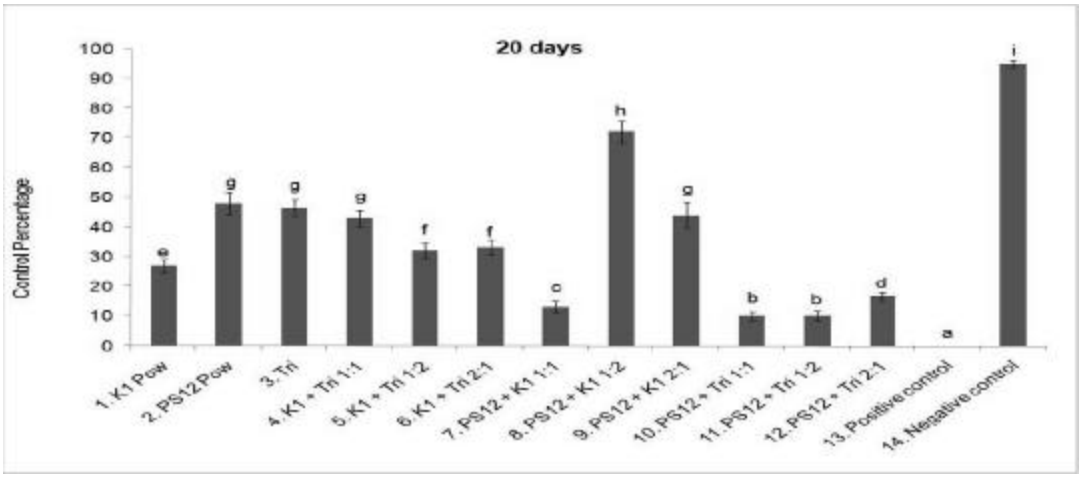
was still best with Ps12 + K1 at 1:2 followed by treatment 4 (K1 + Trisan™ at 1:1) and treatment 12 (Ps12 + Trisan™ at 2:1) ($F = 179.00$, $df = 13$, $p < 0.05$) (Fig. 3b). At day 15, Ps12 + K1 (1:2) still provided >50% control while the other treatments provided poor control of bacterial wilt ($F = 222.20$, $df = 13$, $p < 0.05$) (Fig. 3c).



(a)

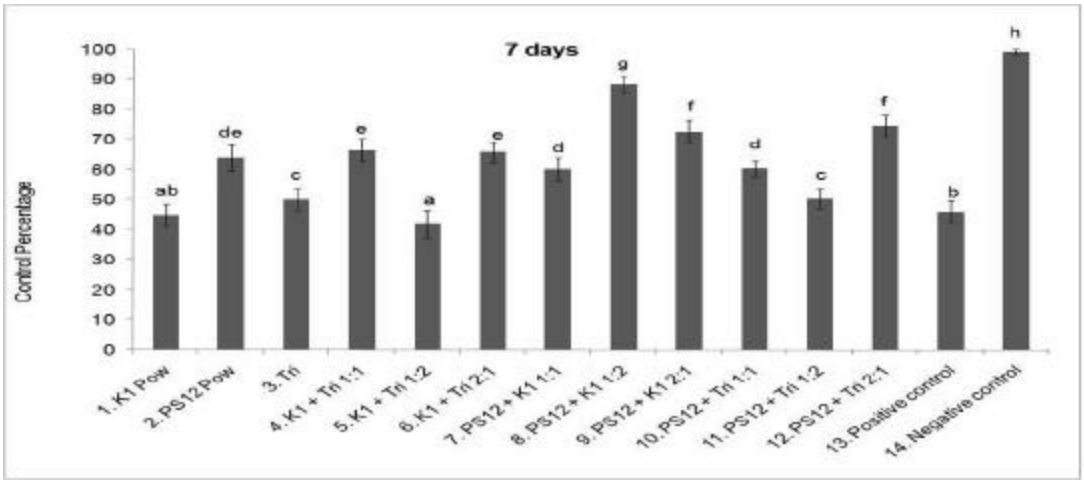


(b)

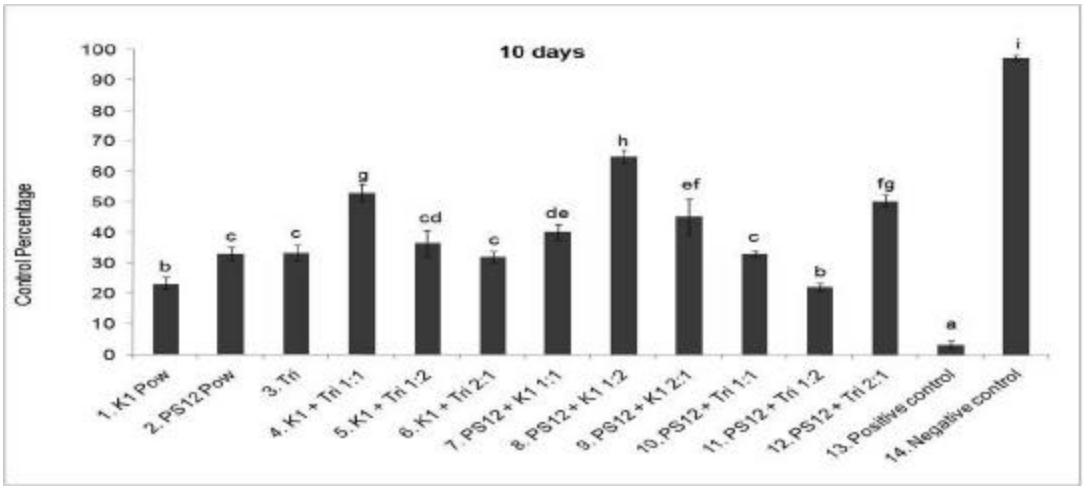


(c)

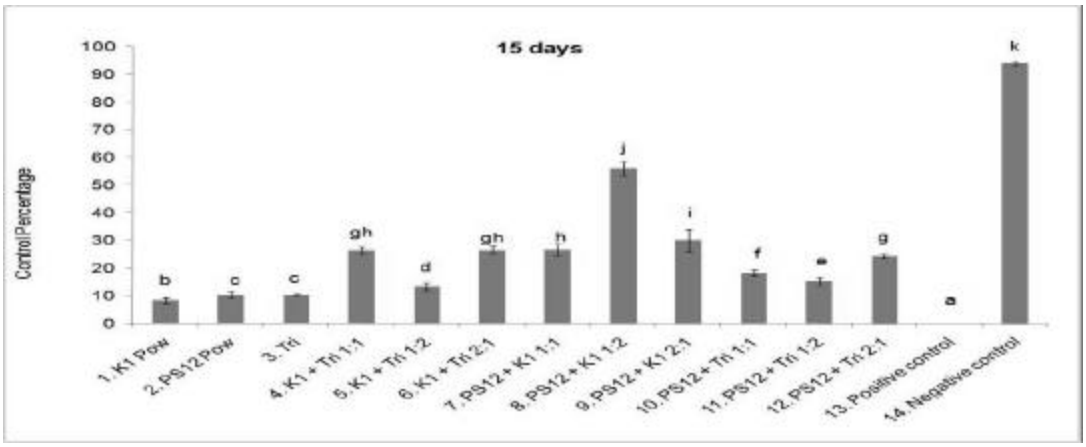
Fig. 2: Effects of antagonists on bacterial wilt of ginger in the greenhouse in the first experiment. Antagonists were added once (before transplanting of ginger) (a. at day 7 - b. at day 15 - c. at day 20)



(a)



(b)



(c)

Fig. 3: Effects of antagonists on bacterial wilt of ginger in the greenhouse in the second experiment. Antagonists were split applied at half amount each (before transplanting of ginger and 7 days later). (a. at day 7 - b. at day 10 - c. at day 15)

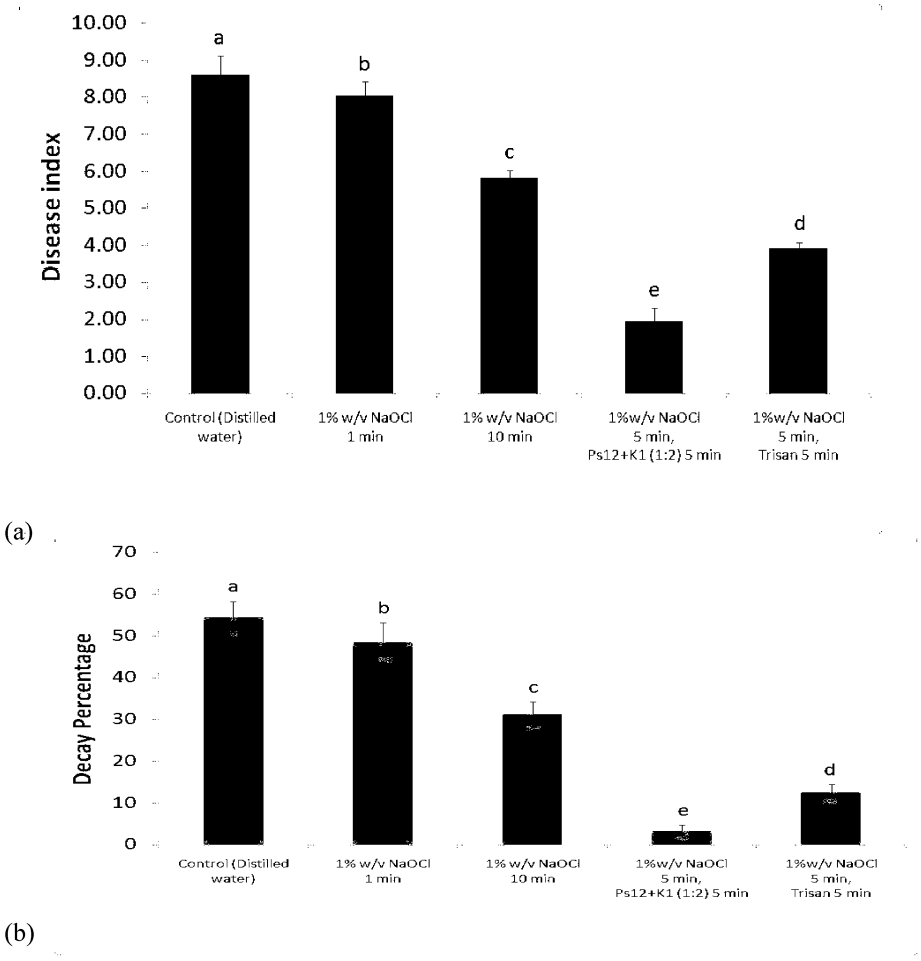


Fig. 4: Disease index and decaying percentage of ginger rhizomes treated with antagonists (a. disease index.- b. decaying percentage.)

Effects of Antagonists on Post Harvest Treatment: Results clearly showed that *R. solanacearum* was not only contaminated on the skin of ginger rhizome but it also penetrated deeply into the ginger tissue through vascular tract (Boonsuebsakul [24]). Fig. 4 showed that dipping the rhizomes in 1%w/v NaOCl solution for 10 min could not prevent the bacterial rot occurred from long term storage (disease index, $F=249.25$, $df=4$, $p<0.05$). The combination of antagonistic bacteria showed a better control than the fungus in protecting the ginger rhizome rot from the pathogen (decay percentage, $F=195.93$, $df=4$, $p<0.05$).

DISCUSSION

The combination of *P. fluorescence* (Ps12) and *B. subtilis* (K1) at a ratio of 1:2 provided the best control of bacterial wilt in the greenhouse experiments, perhaps because *P. fluorescence* can produce siderophores and *B. subtilis* can produce antibiotics. Early in the second greenhouse experiment, however, *T. harzianum* (Trisan^{TM}) combined with *P. fluorescense* (Ps12) provided good control, perhaps because the *T. harzianum* in Trisan^{TM} was able to rapidly colonize the ginger rhizosphere and rhizoplane. Nevertheless, the bacterial combination of *P. fluorescens* and *B. subtilis* provided the best control later in the second greenhouse experiment. As reported in several studies [32 – 36], combinations of multiple antagonists may provide better disease control than single antagonists. Multiple antagonists may enhance the level and consistency of control by providing multiple mechanisms of action and a more stable rhizosphere community and by maintaining efficacy over a wider range of environmental conditions. In particular, combinations of fungi and bacteria may provide protection at different times or under various conditions and occupy different or complementary niches. Such combinations may overcome inconsistencies in the performance of individual isolates.

Our results also indicate that control of bacterial wilt on ginger was better with a single, large application of antagonists at planting than with a split application (half the inoculums applied at planting and half 7 days later). We suspect that this result is consistent with the need to prevent penetration of roots by *R. solanacearum*. Once *R. solanacearum* cells have penetrated through the wounds and into the vascular bundle, antagonists are unlikely to compete with or inhibit the bacteria.

Although a combination of antagonists resulted in better control of bacterial wilt of ginger than single antagonists, the best ratio of antagonists could vary with different genotypes of *R. solanacearum* and with different environmental conditions. Another concern is that the concentrations of *R. solanacearum* were higher in these experiments than in ginger planting areas; a high concentration was used to ensure that those antagonists selected were genuinely effective. Similarly, the pathogen was inoculated onto wounds formed in the stem and root to assure that *R. solanacearum* could rapidly move into the plant vascular system unless blocked by antagonists. Our post harvest treatment procedure using antagonists has recently become acceptable from the importing countries; these include the European communities, Japan, etc. Actually, the bacterial and fungal antagonists have been used together by some exporters in order to protect rhizome rot from fungus for instance *Fusarium oxysporum* [31] as well.

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