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Full Length Research Paper

Carbon adaptation influence the antagonistic ability of Pseudomonas aeruginosa against Fusarium oxysporum f. sp. melonis

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Influences of carbon adaptation on antagonistic activities of three *Pseudomonas aeruginosa* strains V_4, V_7 and V_{10} against *Fusarium oxysporum* f. sp. *melonis* were determined in this study. Results from this study showed that the *P. aeruginosa* strains and their adapted strains significantly inhibited the growth of mycelium of *F. oxysporum* f. sp. *Melonis*, while *in vitro* inhibition of *P. aeruginosa* on the mycelial growth was unaffected by carbon adaptation. In general, the growth of strain V_4 and its antagonistic ability was unaffected by carbon adaptation. However, the growth of the adapted strains V_7-C and V_{10}-C was superior to the corresponding parental strains when root exudates were used as a sole carbon source. In addition, the adapted strains V_7-C and V_{10}-C caused a more decrease in Fusarium infection of melon compared with the corresponding parental strains. Overall, this study revealed that adaptation culture of *P. aeruginosa* strains on carbon-limited media may play an important role in the inhibition of Fusarium wilt of melon seedlings although the effect of carbon adaptation may depend on the test strain.

Key words: Melon, biocontrol, Fusarium wilt, *in vitro*, *in vivo*.

INTRODUCTION

The use of antagonistic bacteria to control soil-borne diseases has been widely explored (Haggag, 2007; Li et al., 2007, 2010; Nwaga et al., 2007; Algam et al., 2010; Charoenporn et al., 2010). In spite of the numerous attempts to commercialize these bacterial strains, only a few have been released as inoculants for agricultural use. The success in formulating microbes depends on *in vivo* efficacy of the inoculum for disease control (Li et al., 2007, 2010). Indeed, the bacteria introduced into soil as agents must be capable of adaptation to a hostile environment (Gu and Mazzola, 2001; Bae et al., 2007).

It has been well known that carbon starvation mediates bacterial resistance to a number of environmental stresses (Van Overbeek et al., 1995; Gu and Mazzola, 2001; Sanin et al., 2003). In particular, several studies reported that composition of the laboratory medium used to prepare inoculum significantly influenced the biocontrol efficacy of antagonistic bacteria (Fuchs et al., 2000; Manjula and Podile, 2001; Shaukat and Siddiqui, 2003; Bae et al., 2007). The relative ability of antagonistic bacteria to grow in nutrient-limited media is also

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Abbreviations: FOM, *Fusarium oxysporum* f. sp. *melonis*; **KMB,** King's medium B; **NA,** nutrient agar; **MM,** minimal medium; **NB,** nutrient broth; **PDA,** potato dextrose agar.

dependent upon prior culture conditions.

In our previous studies, thirty bacterial strains from rhizosphere of melon showed antagonistic activity against *Fusarium oxysporum* f. sp. *melonis* (Fom) (Li et al., 2006), which caused the most destructive disease of melon (Tamietti and Valentino, 2006; Suárez-Estrella et al., 2007; Lopez-Mondejar et al., 2010). The aim of this study was to investigate the influence of carbon adaptation on the growth of three selected *Pseudomonas aeruginosa* strains on different medium and their inhibition activity against Fom *in vitro* and *in vivo*.

MATERIALS AND METHDOS

Bacterial and fungal strains

P. aeruginosa strains V_4, V_7 and V_{10}, which inhibited the *in vitro* mycelial growth of Fom (Li et al., 2006), were used as the parental strains in this study. Fom strain T_{001} isolated from a diseased melon plant was used to evaluate the antifungal activities of antagonistic bacteria.

Adaptation of *P. aeruginosa* strains

P. aeruginosa strains V_4, V_7 and V_{10} were adapted to a carbon-limited environment by repeated culturing on soil agar media amended with a diluted suspension of King's medium B (KMB, Schaad et al., 2001) according to the method of Gu and Mazzola (2001). Three isolates V_4-C, V_7-C and V_{10}-C that possessed a stable colony phenotype were considered to be adapted strains. Sandy soil used in media preparation was obtained from vegetable field in Zhejiang University, China.

Measurement of bacterial growth

After 48 h of pre-culture at 28°C on nutrient agar (NA, Li et al., 2008a, 2009), bacteria were suspended in sterile water and adjusted to a concentration of approximately 1×10^8 CFU/ml. The survival of *P. aeruginosa* parental strains and their adapted strains was conducted in a minimal medium (MM) according to the method of Gu and Mazzola (2001). Furthermore, growth rates of bacterial strains were also assessed in minimal broth or 1/20th-strength KMB broth or 1/20th-strength nutrient broth (NB, Schaad et al., 2001). In addition, the sensitivity of *P. aeruginosa* parental strains and their adapted strains to various forms of environmental stress was compared by incubating them in 1/10th-strength KMB amended with 1 M NaCl to simulate osmotic stress or 8 mM H_2O_2 to simulate oxidative stress. The cell numbers were measured on NA using the plate count method (Li et al., 2008b) after 24 h of incubation. Each treatment had six replicates and the experiment was independently replicated twice.

In vitro antifungal activities

After 48 h of pre-culture at 28°C on NA, the inhibition ability of *Pseudomonas* parental strains and their adapted strains against mycelial growth of Fom was assessed on potato dextrose agar (PDA) according to the methods of Li et al. (2007) and Kazempour and Anvary (2009). Inhibitory effects of cell-free filtrates towards Fom were demonstrated by measuring the diameter of fungal mycelial on PDA containing cell-free filtrates. Cell-free filtrates were obtained by passing bacterial suspension of 10^8 CFU/ml through sterile 0.45 µM-pore-size Millipore filters and divided into two halves, one half was treated with 20 µg/ml proteinase K and the other half was left without proteinase K. A 6-mm-diameter plug from the margin of an actively growing culture of Fom was transferred to the center of a PDA plate, containing 10% cell-free filtrates treated with proteinase K or without. Each treatment had six replicates and the experiment was replicated twice.

Utilization of root exudates

Roots of two-week-old melon seedlings (cultivars blue husk green flesh) grown in sterilized soil were immersed in a 1.5% sodium hypochlorite solution for 20 s and then rinsed repeatedly with sterile water. Melon seedlings were aseptically transferred to a sterile 50 ml triangle bottle containing 30 ml of minimal broth without sucrose. After 14 days of incubation at 28°C with a 12-h photoperiod, melon seedlings were removed and the solution containing root exudates were filtered through a 0.45 µM membrane prior to storing at 4°C until use. Aliquots of root exudates were plated on NA prior to use to check for bacterial contamination, while media exhibiting any form of microbial contamination was discarded. The ability of bacterial strains to utilize root exudates as a sole carbon source was examined according to the method of Gu and Mazzola (2001). Each treatment had six replicates and the experiment was replicated twice.

In vivo antagonistic assays

After surface-sterilization in 1% sodium hypochlorite solution for 2 min, melon seeds were rinsed in sterile water 3 times and then soaked in bacterial suspension (10^8 CFU/ml) for 4 h. Germination of bacterized seeds was determined after incubation on moistened filter paper at 28°C. The autoclaved soil (121°C for 1 h, twice) was infested with Fom barley inoculum at a concentration of 3% (w/w). Barley inoculum was prepared by inoculating Fom T_{001} in autoclaved barley. Soil was dispensed into pots (20 cm diameter; 18 cm height) with a total of six pots per treatment. Bacterized germinated seeds were sown at 15 seeds per pot and the pots were arranged in a randomized manner. Plants were maintained in a greenhouse with a 16 h photoperiod (daytime 16 h at 28°C and nighttime 8 h at 20°C) for four weeks and the experiment was replicated twice. After emergence, 10 seedlings per pot were maintained and watering was done regularly. Stem sections of all seedlings were harvested and surface disinfected as above and placed on PDA to confirm the presence of Fom.

Statistical analyses

The software STATGRAPHICS Plus, version 4.0 (Copyright Manugistics Inc., Rockville, Md., USA) was used to perform the statistical analyses. Levels of significance ($P<0.05$, Fisher's LSD test) of main treatments and their interactions were calculated by analysis of variance after testing for normality and variance homogeneity.

RESULTS

Growth of *P. aeruginosa* strains and their adapted strains

There was no significant difference in colony diameter between *P. aeruginosa* parental strains and their adapted

Table 1. Growth of *Pseudomonas aeruginosa* parental strains and their adapted strains in different medium.

Strain	Colony diameter (mm)	Cell numbers (×10^8 CFU/ml)		
	MM agar	MM broth	1/20th NB	1/20th KMB
V_4	2.1^a	1.1^a	21.0^a	29.3^a
V_4-C	2.0^a	2.0^{bc}	25.3^a	32.0^a
V_7	2.0^a	1.4^{ab}	49.0^{bc}	50.0^b
V_7-C	2.0^a	2.7^c	72.0^d	52.0^b
V_{10}	2.1^a	1.2^a	43.3^b	84.0^c
V_{10}-C	2.1^a	2.4^c	56.0^c	116.7^d

Data from the repeated experiment were pooled and means within a column followed by the same letter are not significantly different (P<0.05, Fisher's LSD test).

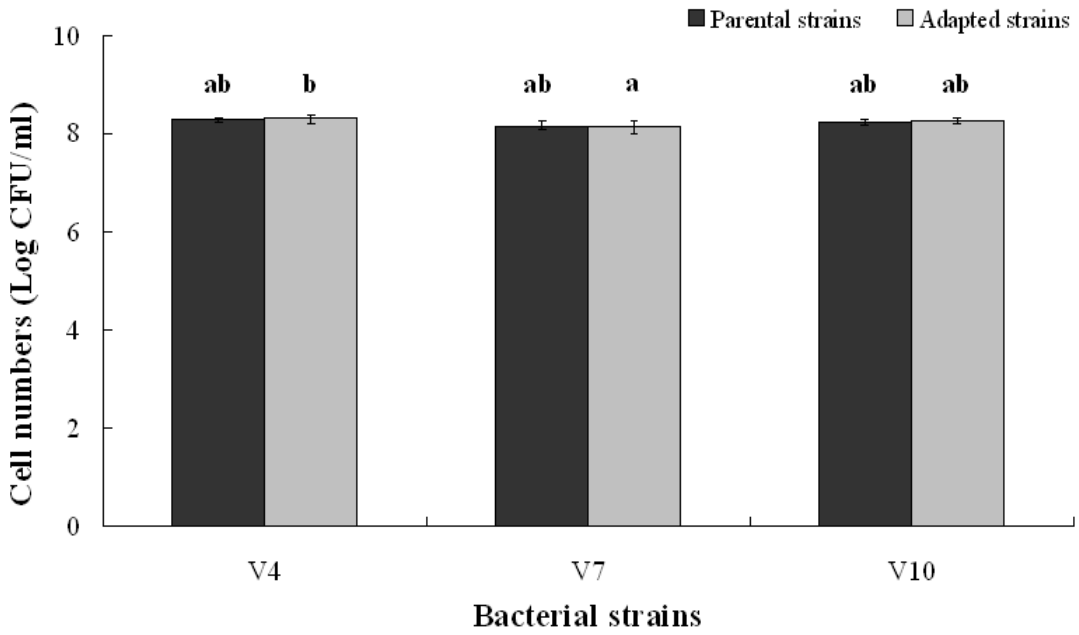

Figure 1. Tolerance to oxidation stress among *Pseudomonas aeruginosa* parental strains and their adapted strains as determined by growth at 28°C in 1/10th KMB amend with 8 mM H_2O_2. Error bars represent standard error and columns with the same letters are not significantly different. (P<0.05, Fisher's LSD test). Strains were established at an initial population of 1.0 ×10^8 CFU/ml.

strains on MM medium (Table 1), which showed that adaptation to carbon-limited conditions did not alter the diameter of bacterial colony on MM agar. However, the growth of the adapted strain V_4-C was superior to the parental strain V_4 in MM broth, but was unaffected in 1/20th strength NB broth and in 1/20th strength KMB broth. The growth of the adapted strains V_7-C and V_{10}-C were also superior to the parental strains V_7 and V_{10} in MM broth and 1/20th strength NB broth, but was unaffected in 1/20th strength KMB broth (Table 1). In addition, the adapted strains V_4-C and V_7-C did not exhibit enhanced tolerance to oxidative stress and osmotic tension significantly as evidenced by growth in 1/10th-strength KMB amended with 8 mM H_2O_2 (Figure 1) or in 1/10th strength KMB amended with 1 M NaCl (Figure 2). The adapted strain V_{10}-C did not cause dramatic changes on the tolerance to oxidative stress (Figure 1), but increased tolerance to osmotic tension (Figure 2).

Utilization of root exudates

There was no significant difference on bacterial growth in minimal media between strain V_4 and the adapted strain V_4-C when root exudates of melon were used as a sole carbon source (Figure 3). In addition, results from this study revealed that the growth of strain V_4 and V_4-C were significantly inhibited by root exudates, which showed that root exudates of melon are unfavorable for the

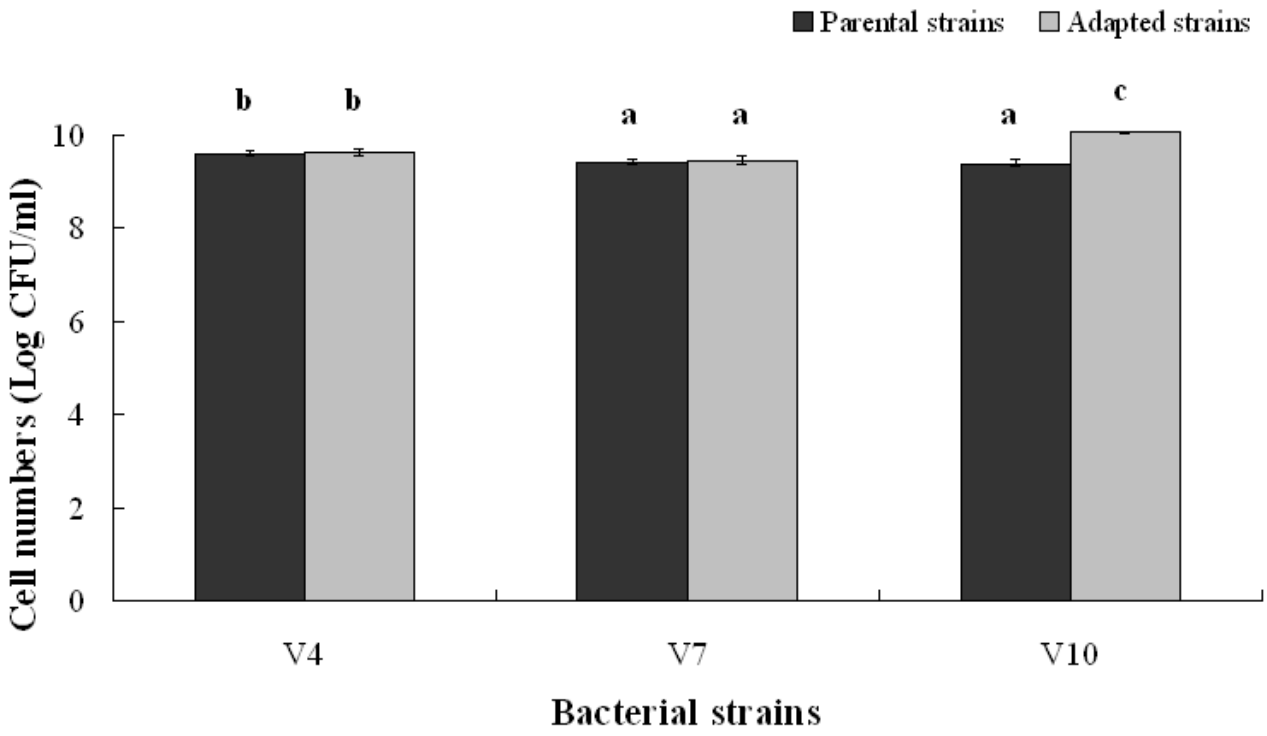

Figure 2. Tolerance to osmotic stress among *Pseudomonas aeruginosa* parental strains and their adapted strains as determined by growth at 28°C in 1/10th KMB amended with 1M NaCl. Error bars represent standard error and columns with the same letters are not significantly different. ($P<0.05$, Fisher's LSD test). Strains were established at an initial population of 1.0×10^8 CFU/ml.

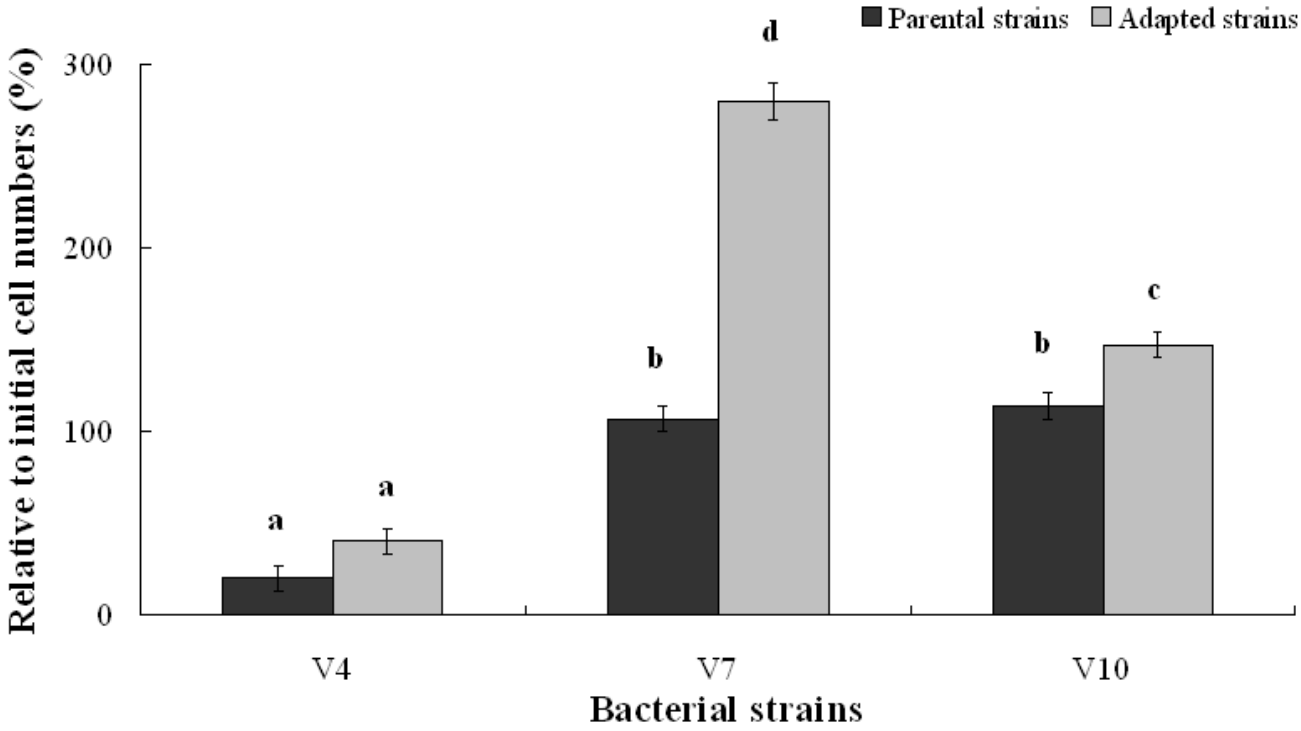

Figure 3. Growth of *Pseudomonas aeruginosa* parental strains and their adapted strains in minimal media containing root exudates as a sole carbon source. Error bars represent standard error and columns with the same letters are not significantly different. ($P<0.05$, Fisher's LSD test). Strains were established at an initial population of 1.0×10^8 CFU/ml.

Table 2. *In vitro* inhibition of the mycelium growth of *Fusarium oxysporum* f. sp. *melonis* by *P. aeruginosa* parental strains and their adapted strains.

Strain	Inhibition percentage of mycelium growth (%)		
	3 day	5 day	7 day
V_4	41.1^{b}	66.0^{c}	75.9^{d}
V_4-C	41.1^{b}	64.8^{c}	76.7^{d}
V_7	31.1^{a}	56.6^{b}	63.3^{bc}
V_7-C	37.8^{b}	56.6^{b}	65.8^{c}
V_{10}	27.8^{a}	49.1^{a}	59.2^{a}
V_{10}-C	27.8^{a}	49.7^{a}	61.3^{ab}

Data from the repeated experiment were pooled and means within a column followed by the same letter are not significantly different (P<0.05, Fisher's LSD test).

Table 3. Effect of *Pseudomonas aeruginosa* parental strains and their adapted strains on seed germination, emergence and biocontrol of Fusarium wilt of melon in greenhouse.

Strain	Germination (%)	Emergence (%)	Wilt incidence (%)	Wilt reduction percentage (%)
V_4	86.7^{ab}	100.0^{a}	30.0^{bc}	52.4^{cd}
V_4-C	90.0^{ab}	100.0^{a}	23.3^{ab}	62.7^{de}
V_7	83.3^{ab}	98.3^{a}	40.0^{d}	33.3^{b}
V_7-C	81.7^{a}	100.0^{a}	33.3^{cd}	47.6^{c}
V_{10}	91.7^{b}	98.3^{a}	33.3^{cd}	46.8^{c}
V_{10}-C	85.0^{ab}	100.0^{a}	16.7^{a}	73.8^{e}
Control	81.7^{a}	100.0^{a}	63.3^{e}	0.0^{a}

Data from the repeated experiment were pooled and means within a column followed by the same letter are not significantly different (P<0.05, Fisher's LSD test). Seedlings uninoculated with *Fusarium oxysporum* f. sp. *melonis* were free of symptoms and not included in statistical analysis.

growth of strain V_4 and its adapted strain V_4-C. Adaptation to carbon-limited conditions did not alter the ability of strain V_4 to utilize root exudates. However, the growth of the adapted strain V_7-C and V_{10}-C was superior to that obtained by the corresponding parental strains when root exudates of melon were used as a sole carbon source (Figure 3).

Inhibition of mycelium growth *in vitro*

Pseudomonas parental strains and their adapted strains showed great inhibition in the mycelium growth and the inhibition percentage increased with the increase of the incubation time in 7 days. In general, there was no significant difference in the *in vitro* inhibition between *Pseudomonas* parental strains and their adapted strains, although, the adapted strain V_7-C showed greater inhibition than the parental strain V_7 after 3 days of incubation (Table 2). In addition, cell-free filtrate of *Pseudomonas* parental strains and their adapted strains significantly inhibited the *in vitro* mycelium growth of Fom. However, there was no significant difference in the *in vitro* inhibition of cell-free filtrate between *Pseudomonas* parental strains and their adapted strains after 7 days of incubation, similar result was obtained when culture filtrate was treated with proteinase K (data not shown).

Inhibition of Fusarium wilts of melon *in vivo*

There was no significant difference between the adapted strains and their parental strains in germination and emergence percentage of melon seeds. In general, the wilt incidence of melon seedlings was significantly reduced by *Pseudomonas* parental strains and their adapted strains compared to the pathogen control. There was no significant difference in the reduction percentage of wilt incidence between the adapted strain V_4-C and the parental strain V_4 although both of them caused a great inhibition in Fusarium wilt of melon. However, the adapted strains V_7-C and V_{10}-C caused a more reduction in wilt incidence of melon seedlings in greenhouse compared to the corresponding parental strains (Table 3).

DISCUSSION

In this study, both *Pseudomonas* parental strains and their adapted strains significantly inhibited the growth of

Fom *in vitro* and *in vivo*. There was no significant difference in antagonistic ability between strain V_4 and its adapted strain V_4-C; in addition, root exudates are unfavorable for the growth of strain V_4 and its adapted strain. However, the adapted strains of V_7 and V_{10} resulted in greater antagonistic effect against Fom compared to the corresponding parental strains, which is consistent with the result that the growth of the adapted strains V_7-C and V_{10}-C was superior to the parental strains when root exudates of melon were used as a sole carbon source. The results obtained from this study partially supported the hypotheses that laboratory cultivation conditions can influence antagonistic ability of rhizobacteria. This result indicate that carbon adaptation enhanced the ability of rhizobacteria to grow in carbon-limited liquid culture relative to the parental strains, which partially revealed the correlation between increase of the adapted strains on growth ability on different medium and increase of the adapted strains on antagonistic ability. However, the all 3 adapted strains did not exhibit greater resistance to osmotic stress and oxidative stress compared to the parental strain except V_{10}-C increased the osmotic stress compared to the parental strain. Therefore, these data is in general different from the result of Gu and Mazzola (2001), which may be attributed to the difference in bacterial species.

The conditions employed in the culture of biological control agents are known to have a significant impact on the production of antimicrobial metabolites, viability in storage, and biocontrol ability even in non-competitive environments such as a sterilized plant growth substrate (Persson et al., 1990; Benhamou et al., 1998; Shaukat and Siddiqui, 2003; Khan et al., 2006; Li et al., 2007). In contrast, results from this study showed that carbon adaptation did not change *in vitro* antagonistic ability of rhizobacteria and its cell-free filtrate against Fom although the mycelium growth was significantly suppressed by both the *Pseudomonas* parental strains and their adapted strains. However, carbon adaptation may improve the biocontrol ability of antagonistic bacteria by increasing their survival in soil environment.

Results from this study also showed that the germination and emergence of bacterized seeds was unaltered by prior cultivation on soil agar. In addition, the inhibition ability of strain V_4 against Fom was unaltered by prior cultivation on soil agar. However, V_7-C and V_{10}-C resulted in a decrease in the incidence of Fusarium wilt relative to the corresponding parental strains, which is consistent with the result that V_7-C and V_{10}-C were superior to the parental strains on root exudates utilization. Therefore, it is likely that the enhanced antagonistic ability of the adapted strains against Fom relative to the parental strains resulted from an altered ability to metabolize root exudates. Bacteria introduced into soil as agents for the biological control of plant pathogens must be capable of adaptation to a hostile environment in terms of both abiotic stress and competition imposed by the resident soil microflora (Gu and Mazzola, 2001; Li et al., 2007, 2010). Numerous studies have been conducted that have sought to enhance the efficacy of biocontrol rhizobacteria through modification of the organism or its environment to promote survival after introduction into the soil environment (Fuchs et al., 2000; Manjula and Podile, 2001; Shaukat and Siddiqui, 2003; Bae et al., 2007; Anvari, 2010). Result from this study indicated that physiological changes induced in response to nutrient limitation can alter nutritional consumption by antagonistic bacteria, which may be of benefit in a soil environment. However, further studies will be necessary to elucidate the survival and stability of these *Pseudomonas* parental strains and their derivatives in soil.

In conclusion, our results clearly demonstrated that adaptation culture of *Pseudomonas* strains on carbon-limited media did not alter the ability of antagonistic bacteria to suppress *in vitro* growth of Fom, but reduced Fusarium wilt infection of melon seedlings *in vivo*, thus supporting the hypothesis that laboratory cultivation conditions can influence the antagonistic ability of bacterial inoculum and may be a means to enhance the efficacy of biocontrol fluorescent pseudomonads. However, the effect of adaptation culture depends on the test strain. In addition, impact of root exudates should be taken into account on the selection of bacterial inoculum.

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