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Full Length Research Paper

Biofilm formation ability of *Paenibacillus polymyxa* and *Paenibacillus macerans* and their inhibitory effect against tomato bacterial wilt

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The potential of biofilm formation of 16 *Paenibacillus* strains and their inhibitory effect against bacterial wilt of tomato seedlings were examined in this study. The crystal violet assay indicated that all strains of *Paenibacillus* except strain MB02-1202 formed biofilm after 96 and 144 h of incubation while there was not significant difference in biofilm formation between strains of *Paenibacillus polymyxa* and strains of *Paenibacillus macerans*. However, the increase level of biofilm formation was associated with the increase of the incubation time and the initial inoculum density. In addition, all *Paenibacillus* strains except strain MB02-428 reduced wilt incidence in tomato seedlings inoculated with *Ralstonia solanacearum* while the cell numbers of *R. solanacearum* in rhizosphere soil was reduced by all *Paenibacillus* strains compared to the pathogen control. In general, most strains of *Paenibacillus* were able to both form biofilm and protect tomato seedlings from bacterial wilt, indicating that biofilm formation may play an important role in the biocontrol of *Paenibacillus*. This is first study regarding the relationship between *in vitro* biofilm formation ability of *Paenibacillus* strains and their inhibitory activity against *R. solanacearum*.

Key words: Biofilm, *Paenibacillus*, *Ralstonia solanacearum*, tomato.

INTRODUCTION

Strains of *Paenibacillus macerans* and *Paenibacillus polymyxa* have been suggested to be involved in plant growth promotion (Jeon et al., 2003; Khan et al., 2008; Zhou et al., 2008), mycorrhizal colonization (Li et al., 2008b) and suppression of pathogens (Akhtar and Siddiqui, 2007; Li et al., 2007, 2008c, 2010; Son et al., 2009). However, applications of antagonistic bacteria required efficient rhizosphere colonization (Siddiqui and Akhtar, 2009a, b). Timmusk et al. (2005, 2009) found that colonization of *P. polymyxa* occurred in the form of biofilm. Therefore, *Paenibacillus* strains with high biofilm formation ability seem to have potential to colonize and inhibit the plant pathogens.

Kim et al. (2009) suggested that biofilm formation is involved in the antimicrobial activity of *P. polymyxa* against plant pathogenic fungi. In addition, their *in vitro* plate experiments indicated that initial density of *P. polymyxa* significantly influences biofilm formation and consequent inhibition of *Phytophthora capsici* (Kim et al., 2009). However, Algam et al. (2010) recently found that the *in vitro* growth of *R. solanacearum* were inhibited by two *Paenibacillus* strains, but unaffected by the other 14 *Paenibacillus* strains, which indicated that *in vitro* antibacterial activities of *Paenibacillus* not only related to the density of *Paenibacillus*, but also depend on the *Paenibacillus* strain.

The objective of this study was to examine the

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relationship between the *in vitro* biofilm formation ability of *Paenibacillus* strains and their antibacterial activity against tomato bacterial wilt.

MATERIALS AND METHODS

Source of *Paenibacillus* strains

Seven strains of *P. polymyxa* and nine strains of *P. macerans* were provided by Department of Integrated Pest Management, Faculty of Agricultural Science, Aarhus University, Denmark, which were isolated from either mycorrhizal or non-mycorrhizal systems (Mansfeld-Giese et al., 2002).

Source of *R. solanacearum*

A virulent strain of *R. solanacearum* Rs-f.91, whose pathogenicity on tomato plants had been confirmed in pre-experiments was obtained from Fujian Academic Agriculture Sciences, China. The pathogenic bacterium was routinely cultured on casamino acids peptone glucose (CPG) agar (Algam et al., 2010) at 28°C for 48 h and temporarily stored in sterile distilled water at room condition.

Biofilm formation of the *Paenibacillus* strains

Biofilm formation were studied in commercially available presterilized, polystyrene, flatbottom 96-well microtitre plates by the method described previously (Lazarevic et al., 2005; Auger et al., 2006) with modification. A single colony was picked up and inoculated into 5 ml of LB broth, and grown at 37°C overnight at 160 rpm. The growth of biofilm formation was initiated by addition of 199 µl of LB broth supplemented with 0·1% (w/v) of glucose and 1 mmol/l $MgSO_4$ to each well of a 96-well microtitre plate. Then, 1 µl of bacterial suspension was inoculated into each well. The microtitre plates were incubated at 30°C without agitation. Biofilm formation was quantified by a crystal violet assay (Lazarevic et al., 2005). The OD_{570} of each well were measured using a Thermo Multiskan EX Microplate Photometer (Thermo Fisher Scientific Inc., Waltham, MA). Each treatment had six replicates and the experiment was repeated twice. Plates without bacteria served as a negative control.

In order to examine the effect of bacterial density on biofilm formation, ten strains of *Paenibacillus* (MB02-226, 428, 429, 454, 513, 523, 727, 1125, 1202 and 1265) were selected and the initial concentration of bacteria was adjusted to OD_{600}=0.20. The 96-well microtitre plates were inoculated with different volumes of each bacterial suspension so as to obtain the final dilutions of 1:2, 1:4, 1:10, 1:20, and 1:200. The formation of biofilm after 24 h of incubation was determined as described above. In addition, the cell growth was evaluated by measuring the OD_{600}. Each treatment had six replicates and the experiment was repeated twice.

Inhibition of tomato wilt

The effect of *Paenibacillus* on tomato wilt was evaluated by a combination of seed treatment and soil drench as described by Algam et al. (2010). Pre-germinated seeds of tomato (cv. Hezou) were sown in plastic pots (10 cm diameter × 10 cm height) containing multi-element nutritional soil (Zhenjiang Lvdao Horticulture Company, China). Two weeks after sowing, plants were inoculated with *R. solanacearum* as described by Algam et al. (2010), and maintained in a temperature-controlled growth chamber with Osram daylight lamps providing supplementary light for a 12 h photoperiod, about 70-80% humidity and 28 ± 2°C. The seedlings were watered with sterile water when necessary. The pots were arranged in a randomized block design with four replicates and one plant per pot. Two months after sowing, plants were harvested and dry weights were determined after drying at 60°C for 3 days. Wilt incidence was calculated as described by Song et al. (2004). In addition, one gram of rhizosphere soil of each treatment was sampled, pooled and dissolved in 10 ml of sterilized distilled water. The soil suspension was then 10-fold serially diluted and inoculated on the CPG medium (Algam et al., 2010). The cell numbers of *R. solanacearum* was determined by the plate count method as described by Li et al. (2008a).

Statistical analysis

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

RESULTS

Biofilm formation in *Paenibacillus*

This study indicated that *P. polymyxa* MB02-226 and MB02-1007 and *P. macerans* MB02-992 were able to form biofilm after 24 h of incubation while *P. polymyxa* MB02-226, MB02-428, MB02-1007, MB02-1172 and *P. macerans* MB02-167, MB02-454, MB02-992 were able to form biofilm after 48 h of incubation (Table 1). However, all 16 *Paenibacillus* strains except *P. polymyxa* MB02-1202 were able to form biofilm after 96 and 144 h of incubation (Table 1). In general, biofilm formation of *Paenibacillus* strains increased with the increase of the incubation time (Table 1) and with the initial bacterial concentration (Table 2), while the final cell density of the *Paenibacillus* strains increased with the increase of the initial inoculum volume (Table 2). To the best of our knowledge, this is first study about *in vitro* biofilm formation in antagonistic bacteria *P. macerans*.

Biocontrol of tomato wilt

Inoculation of tomato seedlings with *R. solanacearum* alone resulted in 95.0% wilt incidence (Figure 1). However, wilt incidence of tomato seedlings were significantly reduced by all 16 *Paenibacillus* strains except strain MB02-428 compared to the pathogen control (Figure 1). The wilt incidence in antagonistic bacterial treatment varied from 5.0 to 70.0% while the maximum inhibition were obtained by *P. polymyxa* MB02-1007, which reduced wilt incidence by 94.7% compared to the pathogen control (Figure 1). No symptoms were observed on the control plant inoculated with sterile water only. There was not significant difference in dry weight of tomato seedlings between the treatments inoculated with *R. solanacearum* alone and the treatments without *R.*

Table 1. Biofilm formation in strains of *Paenibacillus polymyxa* and *Paenibacillus macerans* at different incubation time.

Treatments	OD_{570} at			
	24 h	48 h	96 h	144 h
Control	0.070	0.066	0.067	0.067
P. polymyxa				
MB02-226	0.085*	0.091*	0.085*	0.110*
MB02-376	0.070	0.070	0.090*	0.100*
MB02-428	0.073	0.094*	0.131*	0.103*
MB02-1007	0.084*	0.112*	0.132*	0.156*
MB02-1172	0.070	0.094*	0.152*	0.168*
MB02-1202	0.074	0.071	0.075	0.076
MB02-1265	0.070	0.073	0.122*	0.130*
P. macerans				
MB02-167	0.073	0.080*	0.145*	0.119*
MB02-429	0.073	0.070	0.120*	0.159*
MB02-454	0.073	0.100*	0.129*	0.110*
MB02-513	0.073	0.076	0.119*	0.114*
MB02-523	0.073	0.070	0.243*	0.285*
MB02-727	0.072	0.071	0.113*	0.113*
MB02-992	0.088*	0.089*	0.118*	0.177*
MB02-1125	0.073	0.066	0.153*	0.167*
MB02-1180	0.072	0.075	0.106*	0.098*

A single colony was picked up and inoculated into LB broth, and grown overnight at 37°C and then, one µl of bacterial suspension was inoculated into each well. Data are presented as the means of six replicates from a representative experiment repeated twice with similar results. Means in a column marked with * are significantly different from the control without bacteria according to Fisher's LSD (P = 0.05).

Table 2. Effect of initial density on the growth and biofilm formation of the *Paenibacillus* strains after 24 h of incubation.

Bacterial dilutions	Cells (OD_{600})	Biofilm (OD_{570})	Bacterial dilutions	Cells (OD_{600})	Biofilm (OD_{570})
MB02-226			**MB02-428**		
1:200	0.246 a	0.107 a	1:200	0.223 a	0.103 a
1:20	0.278 b	0.112 ab	1:20	0.268 b	0.112 ab
1:10	0.274 b	0.118 ab	1:10	0.284 bc	0.118 b
1:4	0.289 b	0.120 b	1:4	0.298 c	0.119 b
1:2	0.327 c	0.122 b	1:2	0.338 d	0.121 b
MB02-429			**MB02-454**		
1:200	0.292 a	0.107 a	1:200	0.255 a	0.133 a
1:20	0.297 a	0.116 ab	1:20	0.259 ab	0.138 ab
1:10	0.308 a	0.123 bc	1:10	0.284 bc	0.147 bc
1:4	0.344 b	0.129 cd	1:4	0.292 cd	0.151 c
1:2	0.360 b	0.135 d	1:2	0.311 d	0.165 d
MB02-513			**MB02-523**		
1:200	0.255 a	0.147 a	1:200	0.250 a	0.103 a
1:20	0.259 ab	0.150 ab	1:20	0.291 b	0.112 ab
1:10	0.284 bc	0.152 ab	1:10	0.306 bc	0.114 ab
1:4	0.292 cd	0.161 bc	1:4	0.316 c	0.116 ab
1:2	0.311 d	0.171 c	1:2	0.358 d	0.129 b

Table 2. Cont'd.

MB02-727			**MB02-1125**		
1:200	0.304 a	0.097 a	1:200	0.270 a	0.135 a
1:20	0.312 ab	0.104 ab	1:20	0.289 ab	0.151 ab
1:10	0.311 ab	0.108 ab	1:10	0.301 b	0.172 bc
1:4	0.331 b	0.116 bc	1:4	0.335 c	0.184 cd
1:2	0.367 c	0.126 c	1:2	0.394 d	0.198 d
MB02-1202			**MB02-1265**		
1:200	0.256 a	0.147 a	1:200	0.301 a	0.188 a
1:20	0.255 a	0.158 ab	1:20	0.300 a	0.188 a
1:10	0.273 ab	0.165 b	1:10	0.346 b	0.192 a
1:4	0.290 b	0.172 b	1:4	0.372 c	0.191 a
1:2	0.341 c	0.200 c	1:2	0.592 d	0.255 b

The initial concentration of bacteria is OD_{600}=0.20. Data are presented as the means of six replicates from a representative experiment repeated twice with similar results. Treatments within each strain of *Paenibacillus* with different letters are significantly different (n=6).

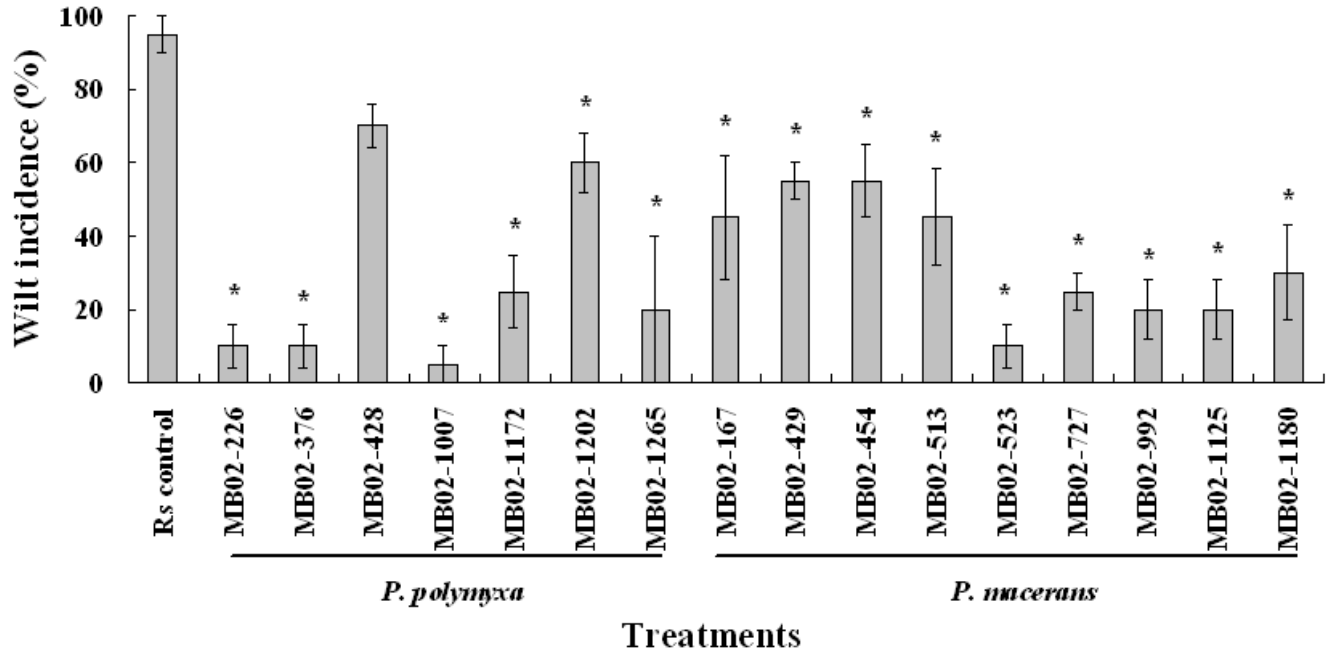

Figure 1. Effect of *Paenibacillus polymyxa* and *Paenibacillus macerans* on wilt incidence in tomato seedlings inoculated with *Ralstonia solanacearum* Rs-f.91. Error bars represent the standard error of the mean and columns marked with * are significantly different from the pathogen control according to Fisher's LSD (*P* = 0.05). Tomato plants uninoculated with *R. solanacearum* were free of symptoms and not included in statistical analysis. Rs = *R. solanacearum*.

solanacearum. In addition, dry weight was unaffected by the 14 *Paenibacillus* strains, but were significantly increased by *P. polymyxa* MB02-1007 and MB02-1172, which caused a 27.4 and 22.6% increase, respectively, compared to the pathogen control (Figure 2).The surviving cell numbers of *R. solanacearum* were 6.86 Log_{10} cfu/g in rhizosphere soil of tomato seedlings inoculated with the pathogen alone (Figure 3). However, the cell numbers of *R. solanacearum* were significantly reduced by all 16 *Paenibacillus* strains compared to the pathogen control (Figure 3). The percentage reduction of *Paenibacillus* strains in the cell numbers of *R. solanacearum* varied from 20.0 to 83.8% while *P. polymyxa* MB02-1007 caused a maximum reduction in the cell numbers of *R. solanacearum* compared to the pathogen control (Figure 3).

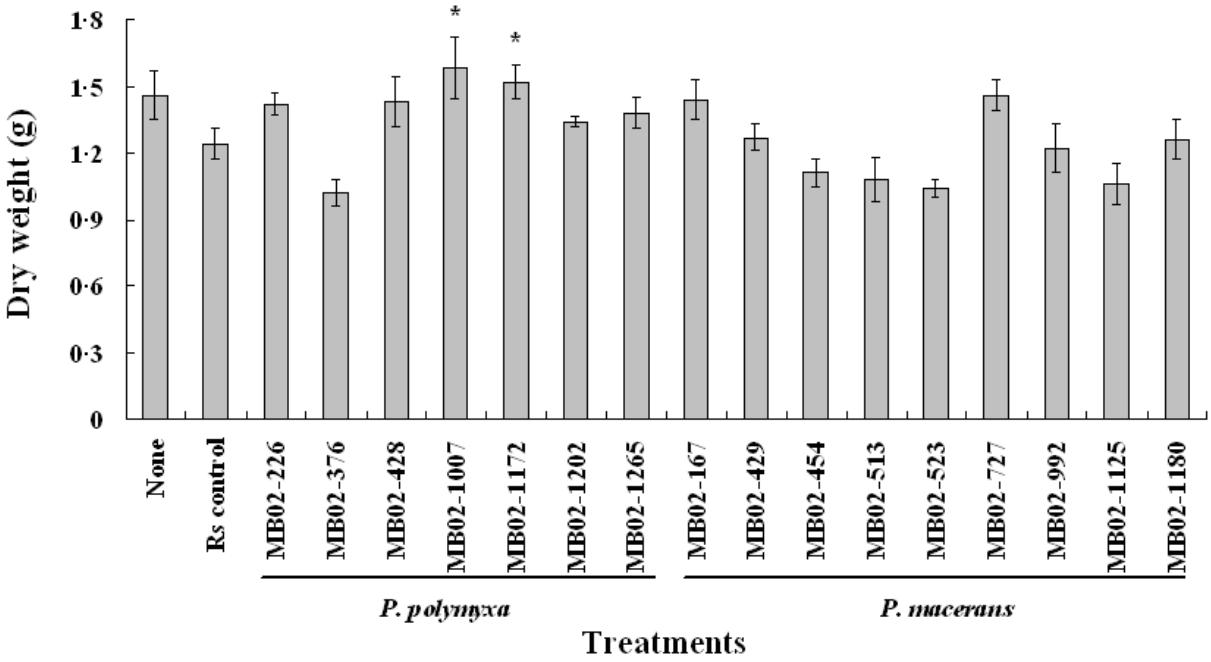

Figure 2. Effect of *Paenibacillus polymyxa* and *Paenibacillus macerans* on dry weight of tomato seedlings inoculated with *Ralstonia solanacearum* Rs-f.91. Error bars represent the standard error of the mean and columns marked with * are significantly different from the pathogen control according to Fisher's LSD (P = 0.05). Rs = *R. solanacearum*.

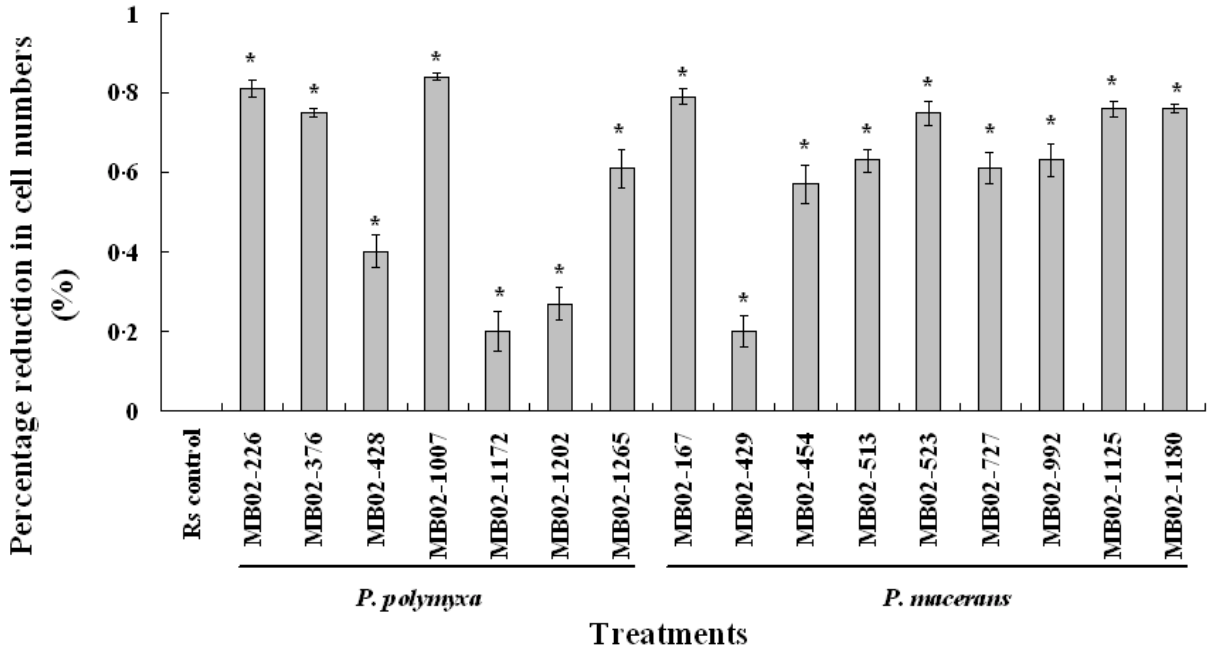

Figure 3. Effect of *Paenibacillus polymyxa* and *Paenibacillus macerans* on the population of *Ralstonia solanacearum* in rhizosphere soil of tomato seedlings. Error bars represent the standard error of the mean and columns marked with * are significantly different from the pathogen control according to Fisher's LSD (P = 0.05). Rs = *R. solanacearum*.

DISCUSSION

In agreement with the result of Algam et al. (2010), *P. polymyxa* MB02-1007 reduced tomato wilt compared to the pathogen control. In addition, the other 14 *Paenibacillus* strains showed *in vivo* antibacterial activity against *R. solanacearum*, which is somewhat different from the result of Algam et al. (2010), who found that 14

out of 16 *Paenibacillus* strains have no *in vitro* inhibitory effect against *R. solanacearum*. However, this result is consistent with the result of Li et al. (2007), who found that none of 16 *Paenibacillus* strains were observed to reduce mycelial growth of *Pythium* while most of the *Paenibacillus* strains showed great potential for controlling *Pythium* damping-off of cucumber.

This study indicated that *P. polymyxa* MB02-428 with biofilm formation *in vitro* were unable to protect tomato seedlings from *R. solanacearum*, but inhibited *Pythium* damping-off in cucumber (Li et al., 2007), while *P. polymyxa* MB02-1202 without biofilm formation *in vitro* protected tomato seedlings from *R. solanacearum*, but did not inhibit the *Pythium* damping-off in cucumber (Li et al., 2007), which revealed that biofilm formation is important, but not enough for the biocontrol of *Paenibacillus* strains. In addition, the role of biofilm formation in antimicrobial activity of *Paenibacillus* strains may be related to the type of the target pathogen.

In agreement with the result of Kim et al. (2009), this study revealed that biofilm formation increased with the increase of the bacterial concentration. In addition, biofilm formation inoculated with different bacterial dilutions is, in general, higher than that inoculated with bacterial suspension incubated overnight. In particular, *P. polymyxa* MB02-1202 could form biofilm inoculated with bacterial dilutions while was unable to form biofilm inoculated with bacterial suspension incubated overnight, which may be attributed to the difference in the initial density of bacteria.

Kim et al. (2009) has suggested that biofilm formation were involved in the *in vitro* antimicrobial activity of *P. polymyxa*. However, our result indicated that most strains of *Paenibacillus* were able to form biofilm after 96 and 144 h of incubation, but did not have the *in vitro* either antifungal activity (Li et al., 2007) or antibacterial activity (Algam et al., 2010). The contrary result may be attributed to the difference in both strains of *Paenibacillus* and the target pathogen. Therefore, the positive correlation between biofilm formation and *in vitro* antimicrobial activity could be limited to the strains of *Paenibacillus* with *in vitro* antimicrobial activity.

Comparing to the other *Paenibacillus* strains, *P. polymyxa* MB02-1007 and *P. macerans* MB02-992 did not have a higher level in biofilm formation after 96 and 144 h of incubation although the *in vitro* antibacterial activity against *R. solanacearum* was observed in the two strains (Algam et al., 2010). However, the two strains could form biofilm while almost all other strains were unable to form biofilm after 24 h of incubation, which revealed a certain incubation time or bacterial concentration is required for biofilm formation in most strains of *Paenibacillus*.

Result from this study indicated that biofilm formation was able to be observed in *P. polymyxa* MB02-226 and MB02-1007 and *P. macerans* MB02-992 after 24 h of incubation. Interestingly, the three strains of *Paenibacillus* not only effectively protected tomato seedlings from bacterial wilt, but also gave a great reduction on *Pythium* damping-off of cucumber in our previous study (Li et al., 2007). Therefore, *Paenibacillus* strains with high biofilm formation ability may be more promise in biocontrol of plant pathogens.

In conclusion, this study indicated that most strains of *Paenibacillus* have the ability both to form biofilm *in vitro* and protect tomato seedlings from bacterial wilt. In general, there was not significant difference in biofilm formation between strains of *P. polymyxa* and strains of *P. macerans*, indicating that biofilm formation maybe depend on the strain itself, but not the species of *Paenibacillus*. The volume of biofilm formation increased with the increase of the incubation time and the cell density. However, the *Paenibacillus* strains with *in vitro* biofilm formation regardless of the incubation time were able to protect tomato from bacterial wilt and cucumber from *Pythium* damping-off. Overall, this data indicated that biofilm formation may play an important role in the biocontrol of *Paenibacillus* strains.

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