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Laboratory Culture Media-Dependent Biocontrol Ability of *Burkholderia gladioli* strain B543

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Cultivation of a biocontrol agent on a certain medium often results in reduced biocontrol efficacy and alters physiological state. In our previous study, *Burkholderia gladioli* strain B543 with long-term subculture on tryptic soy agar resulted in significantly reduced biocontrol ability against cucumber damping-off caused by *P. ultimum*. Therefore, we investigated the influence of laboratory culturing media on biocontrol activity and physiological state of *Burkholderia gladioli* strain B543 by using long-term repeated culture on a certain medium. When isolate B543 were successionally cultured on King's B agar (KBA), tryptic soy agar, nutrient agar (NA), or soil extract agar more than 20 times, the isolate cultured on KBA or NA showed a significantly enhanced biocontrol efficacy and higher population density in the rhizosphere of cucumber compared to that of the others. However, the isolates cultured on KBA more than 20 times showed the lowest production of protease, siderophore, or antifungal substance(s), measured by skim milk agar, Chrome-Azurol-S agar, and potato dextrose agar amended with 10% of the culture filtrate, respectively. Our results suggest that adaptation to proper culturing medium can alter biocontrol ability and physiological state, and we must consider laboratory media in optimizing the use of biocontrol agents.

Keywords : Biocontrol, *Burkholderia gladioli*, cucumber damping-off, culturing media, long-term culturing, *Pythium ultimum*

Many plant growth-promoting rhizobacteria (PGPRs) can enhance plant growth and control plant diseases caused by soilborne plant pathogens when they were used as inoculants (Bae et al., 2004; Gupta et al., 2002; Heungens and Parke, 2000; Hultberg et al., 2000; Jeun et al., 2004; Wei et al., 1996). However, performance of PGPRs for biological control of plant pathogens often varies between field locations and crop seasons (Cook, 1993). Inconsistency of biocontrol agents in controlling efficacy of soilborne diseases may be caused by hostile environments such as abiotic stresses and/or competition with resident soil microflora (Thomashow and Weller, 1996). Furthermore, a part of the inconsistency may be due to degeneration of the biocontrol agent during storage or culturing. Culturing conditions of biocontrol agents are known to have a significant impact on the production of antifungal metabolites (Duffy and Défago, 1999). Gu and Mazzola (2001) reported that adaptation of *Pseudomonas putida* to carbon limited culture conditions by repeated culture enhanced resistance to osmotic tension, oxidative stress, ability of rhizosphere colonization, and improved biological control of Rhizoctonia root rot of apple.

Burkholderia gladiolistrain isolate B543 that was isolated from the rhizosphere of wild spinach was selected for promising its ability to suppress cucumber damping-off caused by *Pythium ultimum*. In previous studies, the isolate showed reduced ability to suppress cucumber damping-off by sub-culturing on tryptic soy agar. Whether long-term repeated culturing of the biocontrol agent on a certain medium could alter its biocontrol ability to Pythium damping-off and physiological state was investigated in this study.

Materials and Methods

Microorganisms. *Burkholderia gladioli* isolate B543 used in this experiment was isolated from the root of wild spinach and selected for biocontrol agent against cucumber damping-off caused by *Pythium ultimum*. Isolate B543^{rif} is a spontaneous mutant of isolate B543 resistant to rifampicin (1 mg ml^{-1}) and was routinely grown on tryptic soy agar medium (TSA, Difco). *Pythium ultimum* and *Phytophthora capsici* were obtained from the fungal and bacterial taxonomical laboratory of Agricultural Science and Technology, Rural Development Administration, Korea.

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Media and cultural conditions of biocontrol agent. Media used in this study were TSA, nutrient agar (NA, Difco), King's B agar (KBA; King et al., 1954), and soil extract agar (SEA; 1 g of peptone, 0.12 g of K_2HPO_4, 0.8 ml of glycerol, 0.12 g of $MgSO_4$, 0.16 g of dextrose, 250 ml of soil extract, 17 g of agar, 750 ml of distilled water). Isolate B543^{rif} routinely grown on TSA was cultured one time or repeatedly over 20 times at 3 day intervals on each above media, respectively. Therefore, they were named as B543-TSA-1, B543-KBA-1, B543-NA-1, B543-SEA-1 for the isolates cultured one time on each media, and B543-TSA-20, B543-KBA-20, B543-NA-20, B543-SEA-20 for the isolates subcultured over 20 times on each media.

Biocontrol ability of medium-dependant isolates against Pythium damping-off. *P. ultimum* maintained on potato dextrose agar (PDA) was cultured on V8 juice broth for 3 days in 23°C. To induce the formation of oospores, the mycelial mass was harvested and placed in sterile distilled water for 2 days in 23°C with light. The oospores were harvested and evenly mixed with potting soil (peat moss and perlite, 7:3 w/w) at the concentration of 5×10^4 oospore/g·soil.

Each medium-dependent isolates of *B. gladioli* isolate B543^{rif} were suspended in sterile distilled water and adjusted at the concentration of approximately 1.0×10^9 colony forming unit (cfu)/ml, respectively. Then, surface-disinfected thirty seeds of cucumber (*Cucumis sativus* L.) cv. Eunsung-bakdadaki (Seminis Co., Korea) were added in the suspension of each medium-dependent isolate. After 1 hour of soaking, the cucumber seeds were sowed in a plastic pot containing potting soil infested with *P. ultimum*. The density of each medium-dependant isolates was aprox. 5.0×10^4 cfu/seed. Treatments were as follows; 1) control without pathogen, 2) untreated control with pathogen, 3) B543-TSA-1 4) B543-KBA-1, 5) B543-NA-1, 6) B543-SEA-1, 7) B543-TSA-20, 8) B543-KBA-20, 9) B543-NA-20, 10) B543-SEA-20. There were three replicates per treatment. All pots were placed in a greenhouse and watered at 4 day intervals. Pre-emergence and post-emergence damping-off were recorded 2 weeks after treatment.

Production of secondary metabolites and biocontrol traits. The production of siderophore, protease, and antifungal substance of each medium-dependant isolates (B543-TSA-20, B543-KBA-20, B543-NA-20, and B543-SEA-20) was investigated in this study. There were five replicates per treatment. Clear zone on the plates was recorded 2 days after incubation at 28°C.

For antifungal activity, each medium-dependant isolates were grown in 1/10 strength tryptic soy broth for 3 days at 28°C. Filtrates were made from the cultures using membrane filters. Then, 10 ml of each filtrates was mixed with 90 ml of half strength of PDA. The mixture was poured into sterile plastic disposable plates. Each plates were placed on the center by mycelium disk (7 mm in diameter) of *P. ultimum* or *Phytophthora capsici* grown on PDA for 7 days. There were five replicates per treatment. All plates were incubated at 25°C and mycelial growth was measured 2 days or 5 days after treatment.

Rhizosphere colonization of medium-dependant B543. The assessment of rhizosphere colonization of each medium-dependant isolates (B543-TSA-20, B543-KBA-20, B543-NA-20, and B543-SEA-20) was examined according to the method of Ahmad and Baker (1985). In brief, cucumber seeds surface-disinfected were soaked in the suspension of each medium-dependent isolates for 1 hr. Then, each cucumber seed was planted in a polyvinyl tube containing a field soil in a plastic container. Then, another plastic container was covered and sealed to maintain soil moisture. All plastic containers were incubated for 3 days in a growth chamber (28°C) with light. There were ten replicates per treatment. The population density of each medium-dependant B543^{rif} in the whole root of cucumber seedling was measured on KBA amended with 100 ug/ml of rifampicin.

Statistic analysis. Analysis of variance of all data except rhizosphere colonization data were performed and the least-significant-difference (LSD, α=0.05) test was used for means separation, using PROC GLM (SAS Institute Inc., Cary, NC). Statistical analysis of colonization data was based on arcsin transformation prior to analysis of variance (p=0.05).

Results

Biocontrol effect of medium-dependant isolates against Pythium damping-off. To examine for the effect of culturing media on biocontrol ability and physiological state of the agent, *Burkholderia gladioli* isolate B543^{rif} routinely grown on TSA was used in this study, which showed reduced biocontrol ability compared to that of original isolate in a previous greenhouse experiment (data not shown). When the isolate B543^{rif} was cultured on TSA, KBA, NA, or SEA at one time, B543-KBA-1 and B543-NA-1 showed a slightly improved biocontrol activity to Pythium damping-off on cucumber compared to that of B543-TSA-1 or B543-SEA-1 (Fig. 1). However, when isolate B543 as successionally cultured more than 20 times, the treatments of B543-KBA-20 and B543-NA-20 revealed a significantly improved biocontrol efficacy compared to that of one time cultured isolates, B543-TSA-20, or B543-

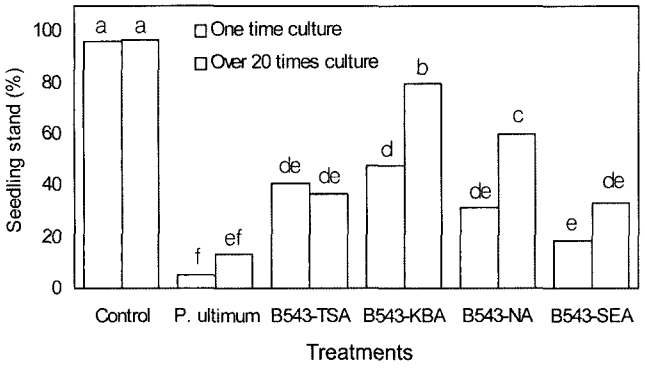

Fig. 1. Effect of medium-dependent *Burkholderia gladioli* strain B543 on the control of cucumber damping-off caused by *Pythium ultimum* when the isolate was cultured at one time (left bar) or over 20 times (right bar) on each media. Means followed by the same letter are not significantly different at $p = 0.05$.

SEA-20, showing the best improvement in biocontrol effect in the treatment of B543-KBA-20.

Production of secondary methabolites and biocontrol traits. The productions of siderophore and protease were examined by measuring clear zone diameter on CAS agar and skim milk agar plates with the filtrates made from 3-day-old Luria-Bertani broth cultures of each medium-dependant isolates. The highest activities of siderophore and protease were achieved in the treatment of B543-TSA-20, while the lowest activities were showed in the treatment of B543-KBA-20 (Table 1).

Table 1. Effect of medium-dependent *Burkholderia gladioli* isolate B543 on the production of siderophore and protease

Treatment	Clear zone (mm)[a]	
	Siderophore	Protease
B543-TSA	17.7a	14.2a
B543-KBA	7.9c	8.5d
B543-NA	9.9b	11.8b
B543-SEA	9.3b	10.1c

[a] Siderophore and protease activity were measured on CAS and skim milk agar plates, respectively. Means followed by the same letter in the column are not significantly different at $P = 0.05$.

Table 2. Antifungal activity of culture filtrate of medium-dependent *Burkholderia gladioli* B543 against *Pythium ultimum* and *Phytophthora capsici* on half strength of potato dextros agar

Treatment	Mycelial growth (mm) of	
	Pythium ultimum	*Phytophthora capsici*
Control	43.3ab	45.4a
B543-TSA	18.0c	20.3c
B543-KBA	44.0a	38.0b
B543-NA	40.6b	35.6bc
B543-SEA	45.0a	38.8b

* Means followed by the same letter in the column are not significantly different at $P = 0.05$.

Table 3. Population density of medium-dependent *Burkholderia gladioli* isolate B543 in the rhizosphere of 3 days cucumber seedlings

Treatment	Initial population density ($\times 10^4$ cfu/seed)	Population density ($\times 10^5$ cfu/g rhizosphere soil)	Percent of increase (%)
B543-TSA	8.27a	0.97b	117.3
B543-KBA	8.30a	2.92a	351.8
B543-NA	3.44b	1.08ab	313.9
B543-SAE	6.37ab	0.71b	111.4

* Means followed by the same letter in the column are not significantly different at $P = 0.05$.

B543-TSA-20 had greastest inhibitory activities against *P. ultimum* and *P. capsici* showing 18.0 mm and 20.3 mm in mycelial growth, while untreated control showed 43.3 and 45.4 mm in the mycelial growth of *P. ultimum* and *P. capsici*, respectively. However, the lowest inhibitory activity resulted in the treatment of B543-NA-20 (Table 2).

Rhizosphere colonization of medium-dependant isolates. When the initial cell density on the cucumber seeds soaked in each bacterial suspension was investigated, the bacterial density was different between medium dependant isolates. Therefore, percent of population increase was calculated for each medium dependant isolated in this experiment. The treatment of B543-KBA-20 resulted in the highest increment (351.8%) of population density in the rhizosphere of cucumber, following 313.9% increase in the treatment of B543-NA-20. However, B543-TSA-20 and B543-SEA-20 showed 117.3 and 11.4% increases, respectively (Table 3).

Discussion

Conditions employed in the culture of biocontrol agents may have a significant influence on the production of antimicrobial substances (Duffy and Défago, 1999), survival in soil, and biocontrol ability (Fuchs et al., 2000; Gu and Mazzola, 2001). We also demonstrate here that cultural media can influence on the biocontrol ability and physiological state of *Burkholderia gladioli*.

In our previous study, the isolate B543 with long-term subculture on TSA resulted in significantly reduced biocontrol ability (approx. 35% reduction compared with original that of the isolate) against cucumber damping-off caused by *P. ultimum* compared to that of the original isolate from the rhizosphere of a plant (data not shown). By using repeated culture more than 20 times on several media

such as King's B agar, nutrient agar, and soil extract agar, we tested if its biocontrol ability against Pythium damping-off was recovered on cucumber. King's B agar and nutrient agar used in this experiment were selected for nutrient rich media, while soil extract agar was chosen for a nutrient poor medium. We also used tryptic soy agar as a control medium. Repeatedly culturing of the isolate over 20 times on TSA (B543-TSA-20) resulted in reduced biocontrol efficacy compared to that of the one-time cultured isolate (B543-TSA-1) on TSA, but no significant difference by statistical analysis, which result coincides with our previous result. King's B agar when the isolate was repeatedly cultured on over 20 times provided recovered or somehow enhanced biocontrol efficacy of *B. gladioli* strain B543 in our study. In fact, we expected that long-term culturing on soil extract agar can result in the recovered biocontrol ability, because cultivation of bacterial agents in a nutrient-limited medium often provides enhanced disease control, survival and adaptation ability to their native habitats (Gu and Mazzola, 2001; Watanabe et al., 2000).

We hypothesized that higher production of antifungal substance including protease or siderophore may result in higher biocontrol efficacy against Pythium damping-off. However, TSA-repeated subculture showed largest production of siderophore and protease, and a significantly inhibitory activity on the mycelial growth of *P. ultimum* and *Phytophthora capsici* than other isolates. With consideration of the above biocontrol results, these results indicate that biocontrol mechanism related to *B. gladioli* strain B543 may not include the production of antifungal substance, siderophore, and protease, which are known as mechanisms in other biocontrol determinants (Duffy and Défago; 1999; Nielsen and Sørensen, 1997; Thomashow and Weller, 1996).

Colonization ability of biocontrol strains in the host rhizosphere has long been considered as a key element to their successful use in the control of soilborne plant pathogens (Bae et al., 1990; Schippers et al., 1987; Weller, 1988). As a result, numerous studies have been conducted to enhance rhizosphere competence of biocontrol agents introduced into soil environments. These studies include genetic modification of biocontrol agents (Roberts et al., 1998), amendment of nutrient (Monne-Loccoz et al., 1999), and introduction method of agents, i.e. soil introduction or seed priming, to enhance root colonization or biocontrol efficacy. Fuchs et al. (2000) demonstrated that composition of the laboratory medium used in the preparation of inoculum significantly influenced the control efficacy of *Pseudomonas fluorescens* against black root rot of cucumber in natural soil microcosms, but not in artificial soil microcosms containing low microbial activity. The difference in disease suppression was not associated with differences in the population of the biocontrol strain in the experimental microcosms, however they did not conducted to differentiate rhizosphere populations of the introduced biocontrol agent. If considering the colonization ability of B543-KBA-20 isolate in the rhizosphere of cucumber, the results obtained in this study might be slightly explained by differences in biocontrol activity of medium-dependent isolates. Further work will be needed to elucidate the mechanisms responsible for this effect.

In conclusion, adaptation of a biocontrol agent to an appropriate culture medium can enhance biocontrol ability and laboratory media must be considered in optimizing the use of a biocontrol agent.

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