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Enhancing the Biological Control of Rice Seedling Disease by Adding Specific Carbon Sources into the *Bacillus cereus* D324 Formulation in Water-Seeded Rice

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Utilization of carbon sources by *Bacillus cereus* D324, a biological control agent, and *Pythium* species, which causes rice seedling disease, was studied with the objective of increasing the efficacy of biological control by providing the biological control agent with specific beneficial carbon sources. D-galactose, D-sorbitol, and D-mannitol were poor carbon sources for *Pythium* spp. growth but were good for *B. cereus* D324 growth. Growth in a growth chamber of rice seeds coated with *B. cereus* D324 amended with specific carbon sources, such as D-galactose and D-sorbitol, showed significantly enhanced seedling emergence compared to seeds coated only with *B. cereus* D324. Field trials showed that both seedling emergence and yield increased, when the above specific carbon sources were added to *B. cereus* D324 in seed coating formulations. This result indicated that amending seed coating formulations with specific carbon sources could significantly increase seedling emergence and yield in the field.

Keywords **:** *Bacillus cereus*, biological control, *Pythium*, rice seedling disease, water-seeded rice

Rice seedling disease, a major problem in water-seeded rice, is caused by several pathogens, including *Pythium*, *Achlya*, and *Fusarium* species. Water-seeded rice (*Oryza sativa* L.) is the most cost-effective for growing rice because it is easier to control weeds and enhance nitrogen use efficiency by maintaining prolonged continuous flooding following water seeding (Groth et al., 1991). However, seedling disease poses a major problem in water-seeded rice, causing stand reduction and growth abnormalities in fields, seedbeds grown for transplanting, and in seed boxes used in mechanical transplanting operations (Rush, 1992; Sung et al., 1983). Soilborne disease control, such as for damping-off, is accomplished by physical or chemical improvements to the characteristics of the soil or cultivating resistance varieties, but mainly by using chemical methods. Chemical methods used to prevent disease, however, can lead to problems of environmental pollution or resistance to chemicals by the long term usage (Lancaster, 1997). Progress is now being made into biological control measures using antagonists of pathogens and supplements that enhance the growth of beneficial soil microorganisms (Baker, 1987; Hornby, 1983).

A great deal of research has focused on the biological control of plant pathogens (Weller, 1988). We have isolated bacterial control agents against *Pythium* species, which could significantly increase rice seedling stands. However, a major limitation of biological control is that the field results obtained are not consistent throughout the year, possibly due to not strong enough for the biological control agents to overcome this inconsistency.

Specific carbon source amendments to biological control agents to reduce postharvest disease of apples caused by *Penicillium* species have resulted in significant increases in the efficacy of biological control (Janisiewicz et al., 1992). It may be possible to afford a competitive advantage to introduced bacterial strains if they are provided with a substrate that can be readily utilized as a carbon source through a seed-coating formulation.

The objectives of the present study were to identify specific carbon sources that can be utilized only by beneficial *B. cereus* D324, and to increase the efficacy of biological control throughout seed coating formulations.

Materials and Methods

Carbon utilization by biological control agents and

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***Pythium* species.** Among bacteria previously tested as biological control agents, *B. cereus* D324 was selected for further study because it demonstrated a high level of competence and strong inhibition of *Pythium aquatile* P140. Comparative carbon source utilization was conducted for *Pythium* species and *B. cereus* D324 used for seedling disease in water-seeded rice. Claus's basal medium (Sneath, 1986) was amended with filter-sterilized (0.25 μm pore size) C^{14} sources (alanine, arabinose, asparagine, cellobiose, galactose, glucose, glycerol, lactose, lactulose, maltose, mannitol, melezitose, proline, and sorbitol) including 0.8 g of KH_2PO_4, 0.2 g of K_2HPO_4, 0.05 g of $CaSO_4 \cdot 2H_2O$, 0.05 g of $MgSO_4 \cdot 7H_2O$, and 0.058 g of NH_4NO_3, and 100 ml of Schmitthener's minor element solution consisting of 30 mg of KH_2PO_4, 30 mg of K_2HPO_4, 20 mg of $MgSO_4 \cdot 7H_2O$, 0.56 mg of $CaCl_2$, 2.88 mg of $MnCl_2$, 1.67 mg of $ZnCl_2$, 0.10 mg of $FeCl_2$, and 11.60 mg of ethylenediaminetetraacetic acid disodium salt (EDTA) in 1 L deionized water. The concentrations of the carbon sources were 5 and 10% for *Pythium* species and *B. cereus* D324, respectively. Three mycelial plugs grown on PV media (Mircetich, 1970) were inoculated into each 125 ml Erlenmeyer flask containing 25 ml of the carbon source broth and incubated at 28°C for 7 days. The mycelia grown were blended for 10 s and the respective absorbances of mycelial suspensions were measured at 600 nm (Mecasys Co., Ltd., Seoul, Korea) after being autozeroed with the respective solution of each carbon source. Single colonies of bacterial strains grown on tryptic soy agar (TSA; Difco, Detroit, MI, USA) were inoculated into 25 ml of basal media supplemented with the respective carbon sources and incubated at 32°C for 72 h. The absorbances of bacterial suspensions were measured at 600 nm after autozeroing with the respective solution of carbon sources. Each treatment had three replications.

Formulation and seed coating. On the basis of carbon utilization, an 8% carbon source suspension of *B. cereus* D324 absorbance of 2.5 at 600 nm was mixed with sterilized talc (v:w=1:1, suspension: talc). The formulation was air-dried at room temperature and pulverized with a mortar and pestle. Rice seeds (cv. Ilpoom) were saturated with 1% guar gum and coated with the talc formulation containing 8% of each individual carbon source (i.e., D-alanine, D-galactose, D-sorbitol, D-mannitol, D-glucose, and D-proline) with the biological control agent and then air-dried at room temperature.

Inoculation and efficacy of different carbon sources in biological control. *P. aquatile* P140 was grown in potato dextrose broth for 7 days and mycelia were harvested, washed with sterile distilled water, and then pulverized with a commercial blender at low speed for 10 s. The absorbance was adjusted to be approximately 1.5 at 600 nm. Glassware (diameter 80 mm×height 120 mm) filled with 120 g soil and 120 ml distilled water was autoclaved for 20 min. Twenty seeds coated with the different formulations described above were planted into each glass container and the mycelial suspension of *P. aquatile* P140 (2 ml per container) was inoculated into each container. In addition, rice seeds were immersed in 200 μg/ml of metalaxyl (Ridomil) fungicide overnight for comparison purposes. The rice seeds planted in the containers were incubated at 28°C in a growth chamber for 2 weeks to determine seedling emergence. The experiment was conducted twice and the data were combined before statistical analysis.

Field experiments. Field trials were conducted to assess the efficacy of biocontrol of seedling disease in water-seeded rice and to compare the effects of fungicide application and *B. cereus* D324 with or without 8% carbon sources on rice seedling establishment and yield. The experiments employed 10 treatments arranged in a randomized complete block with five replications. Plot size was 1 m^2, with a 0.5 m alley separating each of the five replicates. Treatments were base (talc+seed), D-sorbitol, D-galactose, and D-mannitol, with or without *B. cereus* D324, or metalaxyl and untreated seeds. Twenty-five grams of coated seeds with *B. cereus* D324 formulation amended with an 8% carbon source were planted in a 1 m^2 plot in each replication in the rice experimental field of Konkuk Rice Experimental Station, Yeoju, Kyunggido, on 20 May 2005. Seedling emergence was determined on 21 June 2005. Dry weights and water contents of seeds were measured for yield comparison of treatments on 25 October 2005, after harvested panicles were dried for 1 week in a greenhouse. The field trial was repeated from 25 May to 23 October 2006.

Statistical analysis. All experiments were repeated and the data were combined before statistical analysis. Results were analyzed by ANOVA and the pooled mean values were separated on the basis of the least significant difference with LSD or Duncan's multiple range test (SAS 9.01; SAS Institute, Cary, NC, USA). Statistical analysis on seedling emergence in the growth chamber was conducted with arcsine-transformed data.

Results and Discussion

Carbon source utilization by *B. cereus* D324 and *Pythium* spp. Large differences were observed in carbon source utilization between *B. cereus* D324 and *Pythium* (Fig. 1). Of 14 carbon sources, D-mannitol, D-galactose, β-lactose,

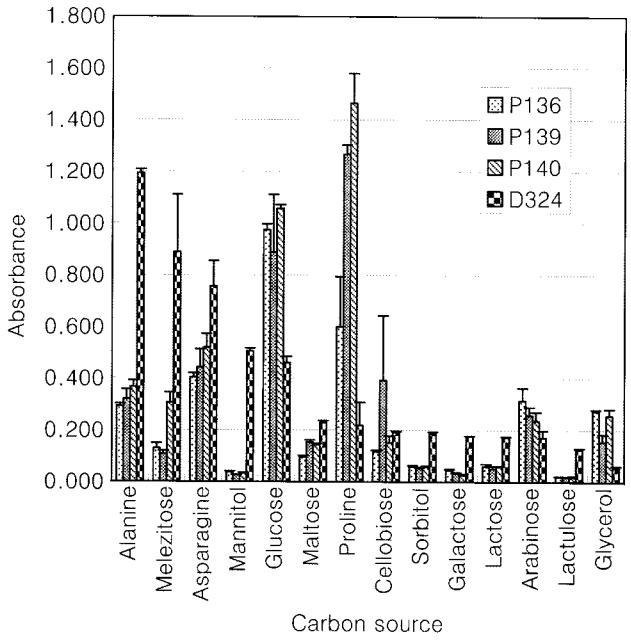

Fig. 1. Comparative carbon utilization by the biological control agent *B. cereus* D324, denoted D324) and three *Pythium* species (denoted P136, P139, P140) *Pythium* 136, *Pythium* 139, *P. aquatile* 140. Absorbances of mycelial and bacterial suspensions were measured at 600 nm after autozeroing with the respective solution of the carbon sources.

D-alanine, L-asparagine, and D-proline were utilized only by *B. cereus* D324 (Fig. 1), although differences in utilization occurred depending on the strain of *B. cereus* (data not shown). D-mannitol and D-galactose were preferred by *Bacillus* species (data not shown) including *B. cereus* strain D324, which was the best performing biocontrol strain against *P. aquatile* strain P140. D-glucose, D-maltose, and L-asparagine were well utilized by *Bacillus* species (data not shown) and all *Pythium* species. Many carbon sources were not utilized by *B. cereus* D324 or *Pythium* (Fig. 1). Based on these results, it might be possible to afford a competitive advantage to an introduced bacterial strain by providing it with a substrate that could be readily utilized as a carbon source via a seed coat formulation.

Effect of different carbon sources on the efficacy of biological control. In the growth chamber study, rice seeds coated with the *B. cereus* D324 formulation amended with D-sorbitol, D-mannitol, and D-galactose had significantly increased seedling emergence (68-74%) compared to untreated seeds (24-36%). Even seeds coated with D-sorbitol, D-mannitol, and D-galactose (Table 1) had significantly increased seedling emergence (60-66%) compared to a base of 36%. However, carbon sources utilized well by both *B. cereus* D324 and *Pythium* did not increase the efficacy of biocontrol compared to *B. cereus* D324 alone (Table 1). Amendment with different carbon sources to the *B. cereus* D324 formulation and even without *B. cereus* D324 significantly increased rice seedling emergence compared to non-amended treatments. This indicates that these carbon sources could be used to increase the efficacy of biocontrol by providing specific carbon sources to be utilized either by an introduced biocontrol agent or by indigenous populations of disease-suppressing microbes on seeds in water-seeded rice.

Table 1. Effect of different carbon sources on the efficacy of biocontrol in rice grown in a growth chamber

Treatment	Seedling emergence (%)±SEM
Untreated seed−Pyth	68.0±4abc[b]
Base[a]+D324+8% Alanine+Pyth	74.0±5a
Base+D324+8% Galactose+Pyth	73.0±6ab
Base+D324+8% Sorbitol+Pyth	69.0±9abcd
Base+D324+8% Mannitol+Pyth	68.0±4abc
Base+8% Sorbitol+Pyth	66.0±2bc
Base+8% Galactose+Pyth	62.0±5bcd
Base+8% Alanine+Pyth	60.0±5cd
Base+D324+8% Glucose+Pyth	57.0±3d
Base+D324+8% Proline+Pyth	55.0±13bcde
Base+8% Mannitol+Pyth	53.0±10de
Base+8% Proline+Pyth	50.0±6de
Base+8% Glucose+Pyth	49.0±3d
Base+D324+Pyth	44.0±10de
Base+Pyth	36.0±9e
Untreated seeds+Pyth	30.0±11e

[a]Base denotes talc and 1% guar gum; D324 is *Bacillus cereus* D324; +Pyth with *P. aquatile* P140 inoculation; −Pyth without *P. aquatile* P140 inoculation.

[b]Means followed by same letters are not significantly different (LSD, $P=0.05$). Statistical analysis was conducted with arcsine-transformed data.

Field trials on the carbon amendment of *B. cereus* D324 on biological control. Seeds coated with *B. cereus* D324 and amended with D-sorbitol, D-mannitol, and D-galactose significantly increased rice seedling emergence compared to untreated seeds in the field. Seeds coated with D-sorbitol and *B. cereus* D324 showed the best seedling emergence, with an increase of 40% compared to untreated seeds. Even seeds coated with D-sorbitol and D-mannitol significantly increased seedling emergence compared to untreated seeds (Fig. 2). Seeds coated with talc and guar gum alone could not increase seedling emergence compared to untreated seeds. However, seed coating treatments with talc, guar gum, and *B. cereus* D324, and with *B. cereus* D324 with specific carbon sources increased seedling emergence significantly more compared to untreated seeds. Treatment

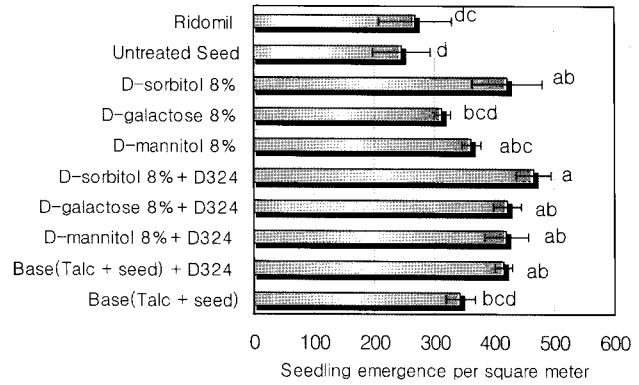

Fig. 2. Effect of carbon sources on seedling emergence in water-seeded rice in the field. All treatments were coated with 1% guar gum with Base (refers to Table 1), except for Ridomil and untreated seed. Error bars indicate the standard error of the mean. Bars with same letters are not significantly different (Duncan's multiple range test, $P=0.001$).

without *B. cereus* D324 did not increase seedling emergence, suggesting that *B. cereus* D324 contributes to increasing seedling emergence in water-seeded rice. Seeds immersed in metalaxyl fungicide did not show increased seedling emergence compared to the untreated seeds, suggesting that metalaxyl treatment through the immersion of seeds was not successful in water-seeded rice in the field.

The treatments that caused significantly higher seedling emergence also produced higher yield. Most of the amendments to *B. cereus* D324 using the beneficial carbon sources described above had significantly higher yields compared to untreated seeds (Table 2). However, no significant difference was observed between treatments with carbon source amendment and *B. cereus* D324, and carbon source amendments without *B. cereus* D324. This does not imply that *B. cereus* D324 has no biological control effect on yield as the carbon source amendments into *B. cereus* D324 and the base+*B. cereus* D324 treatment increased yield significantly more, except in the case with D-mannitol (Table 2). Unexpectedly, no significant difference was observed in seedling emergences between *B. cereus* D324 alone and *B. cereus* plus amendments of beneficial carbon sources in the field, except with D-sorbitol (Table 1). This might have been due to less severe disease development in the field compared to that in the growth chamber. However, we speculate that the amendments could increase the consistency and reliability of biological control activity in the field (Table 1).

Microorganisms isolated from the roots or shoots of a specific crop may be best adapted to that crop and may provide better disease control than organisms originally isolated from other plant species (Cook, 1993; Weller, 1988). Since the root is a major site of infection for *Pythium* or *Achlya* (Chun & Schneider, 1998), colonization and

Table 2. Effect of carbon sources on yield in water-seeded rice in the field

Treatment[a]	Dry weight[c] (g/plot)±SEM
Base[b]	790.8±31.8abc[d]
Base+D324	855.4±37.5a
D-mannitol 8%+D324	829.3±31.6ab
D-galactose 8%+D324	833.8±35.5ab
D-sorbitol 8%+D324	873.0±22.8a
D-mannitol 8%	807.7±27.5ab
D-galactose 8%	792.1±30.7abc
D-sorbitol 8%	791.2±22.6abc
Ridomil	751.0±63.4bc
Untreated seed	705.9±24.0c

[a]All treatments except for Ridomil (metalaxyl) and untreated seed were coated with 1% guar gum.
[b]Base denotes talc and 1% guar gum; D324 is *B. cereus* D324.
[c]Dry weight and water content of seeds was measured for yield comparison of treatments on 25 October 2005, after panicles were dried for a week postharvest in a greenhouse.
[d]Means followed by same letters are not significantly different (LSD, $P=0.0619$).

protection of roots by a potential biocontrol agent may be important for the control of seedling disease in rice. Bacterial strains isolated from rice stems have also been used as potential biological control agents for rice seedling disease caused by *Pythium* or *Achlya*, leading to significant increases in seedling height and dry weight compared to non-inoculated seeds (Adhikari et al., 2001).

We demonstrated that amendments with specific carbon sources could increase the efficacy of biological control but have not elucidated the mechanism of that increased efficacy. Chun (1997) suggested that biological control might not be attributable to an increased population of the introduced biological control agent but rather to a quantitative or qualitative change in the total microbial population associated with germinating seeds. Moreover, the altered development of rhizosphere communities on *B. cereus* UW85n1 treated roots might have a potential relationship with disease suppression (Gilbert et al., 1996). Janisiewicz et al. (1992) studied nutritional enhancement of biological control against blue mold on apples. In their work, nitrogen sources that were preferentially utilized by the biocontrol agents reduced disease severity when applied to the biological control agents. However, preferentially utilized carbon sources did not reduce lesion size caused by the blue mold fungus, and these authors suggested that carbon substrates were not limiting in the infection court since they were already abundant. Specific carbon sources may influence the expression of specific antifungal metabolites of uncharacterized microbes associated with germinating rice seeds.

Our findings clearly show that differences occurred in

carbon utilization between *B. cereus* D324 and *Pythium* species and that the efficacy of biological control could be increased through amendments with specific carbon sources utilized only by the biological control agent (*B. cereus* D324) to control rice seedling disease in water-seeded rice.

Acknowledgment

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References

Baker, K. F. 1987. Evolving concepts of biological of plant pathogens. *Annu. Rev. Phytopathol.* 25:67-85.

Chun, S. C. 1997. Etiology and Biological Control of Rice Seedling Disease in Water-Seeded Rice. Ph. D. dissertation. Louisiana State University. 104 pp.

Chun, S. C. and Schneider, R. W. 1998. Sites of infection by *Pythium* species in rice seedlings and effects of plant age and water depth on disease development. *Phytopathology* 88:1255-1261.

Cook, R. J. 1993. Making greater use of introduced microorganisms for biological control of plant pathogens. *Annu. Rev. Phytopathol.* 31:53-80.

Gilbert, G. S., Clayton, M. K., Handelsman, J. and Parke, J. L. 1996. Use of cluster and discriminant analyses to compare rhizosphere bacterial communities following biological perturbation. *Microb. Ecol.* 32:123-147.

Groth, D. E., Rush, M. C. and Hollier, C. A. 1991. Rice Diseases and Disorders in Louisiana. Louisiana Agric. Exper. Sta. Bull. No. 828. 37 pp.

Hornby, D. 1983. Suppressive soils. *Annu. Rev. Phytopathol.* 21: 65-85.

Janisiewicz, W. J., Usall, J. and Bros, B. 1992. Nutritional enhancement of biocontrol of blue mold on apples. *Phytopathology* 82:1364-1370.

Lancaster, M. E. 1997. A newsletter for commercial vegetable growers. Agricultural extension agent. Volume. Issue 4.

Mircetich, S. M. 1970. The role of *Pythium* in feeder roots of diseased and symptomless peach trees and in orchard soils in peach tree decline. *Phytopathology* 61:357-360.

Rush, M. C. 1992. Seedling diseases. In: Compendium of Rice Diseases. R.K. Webster and P.S. Gunnell, eds. 1992, pp. 12-13. American Phytopathological Society, St. Paul, MN. 62 pp.

Sneath, P. H. A. 1986. Endospore-forming Gram-positive rods and cocci. In: *Bergey's Manual of Systematic Bacteriology.* Vol. 2. Williams and Wilkins, Baltimore, pp. 1104-1207

Sung, J. M., Jin, K. S., Lee, S. C. and Park, J. S. 1983. Identification and pathogenicity of *Pythium* spp. associated with seedling damping-off of rice. *Kor. J. Mycol.* 11:27-32. (In Korean).

Tika, B. A., Joseph, C. M., Guoping, Y., Donald, A. P. and Louise, M. N. 2001. Evaluation of bacteria isolated from rice for plant growth promotion and biological control of seedling disease of rice. *Can. J. Microbiol.* 47:916-924.

Weller, D. M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annu. Rev. Phytopathol.* 26:379-407.