Synergistic Effect of Photosynthetic Bacteria and Isolated Bacteria in Their Antifungal Activities against Root Rot Fungi

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Antifungal bacterial (AB) in root rot fungus (RRF)-contaminated sweet potato farms were isolated, and seven strains were initially chosen as antagonistic candidates. An antagonistic test by using the mycelial disk placement method revealed that one AB strain by itself could inhibit the RRF growth. This AB strain was identified as Bacillus polyfermenticus based on phylogeny of 16S ribosomal RNA genes. Two AB strains (Bacillus aerophilus) displayed high levels of antifungal activity when paired with photosynthetic bacterial strain A (a purple non-sulfur photosynthetic bacterium Rhodopseudomonas faecalis). The results suggest the possible use of the isolates as agents for the biological control of the RRF infection of agricultural products in fields of cultivation.

Key words: Purple non-sulfur photosynthetic bacterium / Root rot fungi / Rhodopseudomonas / Bacillus / Biocontrol.

In a previous study, a purple non-sulfur photosynthetic bacterial strain was isolated from swine sewage wastewater. Effects on wastewater treatment were investigated by applying this strain (Wei et al., 2016). Phylogenetic results showed that it was closely related to Rhodopseudomonas faecalis. On the other hand, purple non-sulfur photosynthetic bacteria have been reported as versatile microorganisms that promote growth and enhance the yield of plants (Kobayashi et al., 1971, Elbadry et al., 1999). Furthermore, applying photosynthetic bacteria as organic fertilizer might improve the growth of microorganisms which produce antibiotics, consequently suppressing the growth of pathogenic fungi (Kitamura et al., 1984).

Diverse plant diseases are caused by phytopatho-
genic filamentous fungi. Among them, the violet root rot disease, caused by different species of the genus *Helicobasidium*, has prevailed around the world (Nakamura et al., 2004). Different strategies have been tested to reduce their impact, including soil cultivation, invasion prevention, chemical and biological control (biocontrol), and use of resistant rootstock (Coffey and Guillemet, 1987; Cotterill, 1993; Erwin and Ribeir, 1996). Biological control appears promising due to its friendliness to the environment (Pratibha et al., 2012).

Numerous microorganisms isolated from different soil samples and host plants have been found to produce antibiotics in vitro (Raaijmakers et al., 2002). They include many representative genera, such as the genera *Bacillus* and *Trichoderma* which are among the most studied (Weller, 1988, Handelsman, 1996). Antibiotic production by marine photosynthetic bacteria has also been reported (Burgess et al., 1991). On the other hand, using a combination of biocontrol species could be one approach to improve the performance of a biological control. Duffy et al. (1996) suggested that mixture of *Trichoderma* and fluorescent *Pseudomonads*, compared to individual agents, resulted in substantially better control of the take-all root disease in wheat. Currently, there is limited research focused on the antifungal effects of photosynthetic bacteria (PSB), especially in combination with other bacterial strains.

In Kagoshima Prefecture, the amount of sweet potato harvested ranks first in Japan. However, during production, plant diseases have brought problems such as farmland desertion, low product quality, and agricultural yield decrease, and most of these diseases are caused by pathogenic fungus. Among them, the violet root rot caused by *Helicobasidium mompa* is an important disease of sweet potatoes. Crop rotation and fungicide have been applied but have not provided consistent control on a commercial scale. The isolated photosynthetic bacterial strain has been applied on the farm as a fertilizer and suppressed the growth of violet RRF, but the scientific mechanism remained unknown. In addition, fungus growth-inhibiting rhizobacteria was also isolated from the infected farm. This study was initiated because of a lack of information about the ability of PSB and its combination with other strains to suppress violet root rot fungus (RRF) in Kagoshima Prefecture, Japan. Thus, the aim of this study is to establish a practical method for the biological control of the violet root rot disease affecting the farming of sweet potatoes.

Soil samples and sweet potato samples were collected from an infected sweet potato farm land (Kanoya City, Kagoshima Prefecture, Japan) in February 2012. After collection, all samples were transferred to the laboratory and stored at 10°C until the isolation of microbes could commence.

A photosynthetic bacterial strain (PSB strain A) was deposited in the Microbiology Laboratory, Faculty of Fisheries, Kagoshima University and stored at 10°C for maintenance. For experimental application, the PSB was transferred into 30 ml of the Basic I liquid medium (Kitamura et al., 1984). After incubation at 25°C under dark conditions for 24 h, the culture was incubated at 30°C for 14 d under 12/12 light/dark cycles.

A pathogenic RRF was isolated by the agar plate dilution method using the oatmeal medium (Nakamura, 2009). Mycelia on the surface of the rhizomes were cut off and transferred into 1 ml of water. After shaking the mixture vigorously, a 10-fold serial dilution was applied, and 100 μL of the dilution solution was added to the agar medium. The plates were cultivated at 25°C for 7 d, and the obtained colonies were isolated.

Bacteria were isolated from the infected farm soil by the plate-spreading method using a nutrient broth (NB) plate medium (1% polypeptone, 0.5% meat extract, 0.2% NaCl, pH 7.0-7.2). One gram of the soil sample was suspended into 1 ml of water, and the suspension was serially diluted 10-fold. A hundred microliter of the dilution was spread onto the agar plates and cultivated at 25°C for 7 d to isolate the colonies. Representative colonies of RRF and bacterial strains were re-streaked and cultivated on new agar plates. All the isolates were stored at 10°C for further analysis.

Colonies of the microorganisms were obtained by streaking and cultivating the isolates on the agar plate media. Optical microscopic observation was carried out by applying the liquid culture of the microorganisms while observation by DAPI staining was also conducted. DNA of the isolated microorganisms was extracted from the liquid cultures by using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The extracted DNA was used for PCR amplification of 16S ribosomal RNA genes (16S rDNA). PCR products were collected and purified as described by Wei et al. in a previous study (Wei et al., 2016).

Nucleotide sequences of the PCR-amplified 16S rDNA were determined with the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, USA). The obtained sequences were assembled with the program DNASIS Pro Version 2.7 (Hitachi Solutions, Tokyo, Japan).

Closely related sequences were obtained from the GenBank DNA database by using the basic local alignment search tool (BLAST) program. Phylogenetic analysis with the neighbor-joining method was conducted using the program MEGA Version 5 (Tamura et al., 2011). The nucleotide sequences of the partial sequence of the 16S rDNA determined in this study have been submitted to the DDBJ database under the accession numbers LC092191, LC092192, LC092193.
Selection of the isolated PSB and AB was conducted according to Cassandra et al. (2004). A hundred microliters of the RRF culture, cultivated in the oatmeal liquid medium at 25°C for 14 d, was inoculated on the oatmeal agar medium. Sterilized filter disks with a diameter of 5 mm, perforated from Advantec No.2 filter paper (Toyo Roshi Kaisha, Co., Ltd., Tokyo, Japan), were placed on the agar plates. Isolated AB strains were incubated in NB medium at 25°C for 3 d, while PSB was incubated in Basic I liquid medium at 30°C for 14 d, then 10 µL of the microbial culture was spotted onto the disks. The test plates were incubated at 25°C and the inhibition zones around the filter disks were observed after 14 d cultivation.

Potential candidates antagonistic to the RRF which were screened by the preliminary test were further tested using a modified mycelial disk placement (MDP) method (Shimane and Takahashi, 1993). Sterilized filter disks with a diameter of 5 mm were placed on each oatmeal agar plate and spotted with 10 µL of the culture of the antagonists. To investigate the synergistic effects, both AB cultures and their mixture with PSB were applied. Mycelial disks with a diameter of 5 mm were cut from the surface of the RRF-cultivated agar plates and overlaid on the filter disks. The plates were incubated at 25°C and the antagonistic activity was evaluated on the basis of suppression of the mycelial growth.

Isolation of microorganisms was conducted using infected soil and sweet potato samples. After the 7 d incubation on the agar plates, many colonies were observed on the agar plates. The typical colonies were picked up, and finally one RRF infecting the rhizomes of sweet potato samples and 11 bacteria strains inhabiting the soil of sweet potato fields were isolated from the rhizome and the soil samples, respectively. The bacteria isolates showed similar cell morphology, the cells were short rod-shaped with similar sizes. On the other hand, RRF showed morphological features of the violet root rot fungal species, Helicobasidium mompa, i.e., mycelia with a light red color were extended over the agar plates (Fig.1A), and mycelial structure was observed microscopically (Fig.1B).

Phylogenetic analysis of the AB isolates revealed that three strains, AUT3, 8, and 9, were all the members of the genus Bacillus (Fig.2). In addition, AUT3 was closely related to Bacillus polyfermenticus, while AUT8 and 9, which had almost the same 16S ribosomal RNA gene sequences, were relatives of Bacillus aerophilus. Isolates of the genus Bacillus are known as antagonists
The antagonistic activity of the isolated PSB and AB to RRF was compared by inoculating their liquid culture onto the paper disks placed on the fungus-cultivated agar plates. The AB strains AUT3, 5, 7, 8, 9, 10, and 11 showed antagonism in 14 d (data not shown). Thus, these strains were selected for further analysis of antagonism. Although the PSB strain showed limited growth-inhibiting zones of RRF in 7 d, PSB was applied in the following experiment to investigate its synergistic effects on antifungal activities.

The results of antagonistic test using the MDP method are shown in Figs. 3, 4, and summarized in Table 1. After 5 d cultivation, the test plates to which the AB strains AUT5, 7, and 10 were inoculated showed RRF growth, although RRF also grew on AUT8, 9, and 11 in 10 d (data not shown). On the other hand, AUT3 completely impeded the RRF growth (Fig. 3C, 2AUT3; Figs. 4A-1), suggesting a high antagonistic effect. Interestingly, the PSB strain synergistically suppressed the RRF growth: although the AB strains AUT8 and 9 were only slightly antagonistic (Fig. 3C, 5AUT8 and 6AUT9; Figs. 4B-1 and 4C-1), the addition of PSB led to the complete growth inhibition of RRF (Fig. 3C, 12PSB+AUT8 and 13PSB+AUT9; Figs. 4B-2 and 4C-2).

Though AUT3 could suppress RRF growth by itself, application of a single biocontrol strain has limited tolerance to changes in environmental conditions (Weller et al., 2016).

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| Treatment No. | Microorganisms | Antagonistic Effect* |
|---------------|----------------|---------------------|
| 1             | PSB            | –                   |
| 2             | AUT3           | +                   |
| 3             | AUT5           | –                   |
| 4             | AUT7           | –                   |
| 5             | AUT8           | –                   |
| 6             | AUT9           | –                   |
| 7             | AUT10          | –                   |
| 8             | AUT11          | –                   |
| 9             | PSB+AUT3       | +                   |
| 10            | PSB+AUT5       | –                   |
| 11            | PSB+AUT7       | –                   |
| 12            | PSB+AUT8       | +                   |
| 13            | PSB+AUT9       | +                   |
| 14            | PSB+AUT10      | –                   |
| 15            | PSB+AUT11      | –                   |

*Antagonistic effect was evaluated from the formation of the blank area through a modified mycelial disk placement (MDP) method.
al., 1994). On the other hand, disease control has been achieved using the combination of *Pseudomonas*, *Bacillus* and *Trichoderma* strains or other combinations of biocontrol species (Latha et al., 2011, Kamal et al., 2009). Although PSB did not show any antagonistic activity by itself in this study, the results suggested that PSB might be able to intensify the inhibitory effect on the RRF growth. Furthermore, PSB contain a number of enzymatic conversions that may yield economically valuable products and improve the growth of photosynthetic plant (Fuller, 1995, Sasaki et al., 1987). Thus, combining biocontrol strains should be suggested to enhance the level and consistency of antifungal effects. Further studies should be conducted to elucidate the mechanism and optimize the antifungal effects for practical use.

This study clarified the antagonistic characteristics of the AB as potential agents for the biological control of the pathogenic root rot fungus. The synergistic antifungal activities of AB and PSB suggest that applying a variety of antibiotics and other useful microorganisms to treat root rot fungus should be considered. In the presence of PSB, the secretion of antibiotics by *Bacillus* species was somehow intensified. Chemical and biological analysis should be conducted to investigate the mechanism of this cooperative action. Furthermore, the antibiotics should be identified using chemical techniques, such as chromatography. The findings of this study might be very significant from the viewpoint of biological prevention against and control of the root rot disease of cultivated sweet potatoes.

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