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Screening of endophytic bacteria against fungal plant pathogens

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Abstract

Bacterial endophytes were found from 6 plant leaves among 35 plant leaves screened. Two of the isolated bacteria showed antagonistic activity against fungal plant pathogens. An isolate named KL1 showed the clear inihibition against plant pathogens, *Fusarium oxysporum* and *Rhizoctonia solani*, on PDA as well as TSA plate. Supernatant of the bacterial culture also showed the clear inhibition against the fungal growth on the plate and the antibiotic substance was identified as iturin A by HPLC analysis. KL1 was identified as *Bacillus* sp. from the 16S rRNA gene analysis. Very thin hyphae of *R. solani* was miccroscopically observed when the fungus was co-cultivated with KL1.

Key words: biocontrol; endophyte; plant pathogens

Introduction

Agriculture is a main industry to produce food, and the bacteria in soil assumed to be involved in the role of efficient food production. On the other hand other microbes such as *Fusarium* and *Rhizoctonia* species are widely distributed soil-borne pathogens and cause diseases on a wide range of economically important plant species (Garcia et al., 2006). *Rhizoctonia solani* is known to reduce the production of crops such as rice (Mew and Rosales, 1986), cucumber (Trillas et al., 2006) and lettuce (Grosch et al., 2011). Strategies to control *Rhizoctonia* diseases are limited and the disease is spreading all over the world. *Fusarium oxysporum* also invades roots and causes wilt diseases through colonization in xylem tissue of host plants, and reduces the production of crops. Synthetic chemical fungicides have long been used as active agents in reducing the plant diseases. However, they are costly, can cause environmental pollution, and may induce drug-resistant pathogens. Considering the limitations of chemical fungicides, it seems appropriate to search for a supplemental control strategy. Biological control, or the use of microorganisms to prevent plant diseases, offers an attractive alternative or supplement to chemical pesticides (San-Lang et al., 2002).

Therefore, developing new biological control agents using antagonistic bacteria seems to be an ideal solution for this problem. We reported that endophyte have the potential of used as new isolation source of biocontrol agent.

1 Materials and methods

1.1 Plant leaf samples

Endophytic bacteria were isolated from 35 different plant leaf samples in Wakayama prefecture in Japan.

1.2 Screening of endophytes

Surface of the leave samples was disinfected in 70% ethanol for 60 sec, followed by 1.0% NaOCl for 5 min, and 70% ethanol for 30 sec, thoroughly washed with sterile distilled water. After sterilization, leaf samples were cut into a 5-mm square and put on the potato dextrose broth agar medium (PDA) and trypto-soya broth agar medium (TSA), and incubated at 24°C or 30°C, respectively. Isolated endophytic bacteria were assayed for antifungal activity against *F. oxysporum* or *R. solani* on PDA and TSA plates.

1.3 Antifungal activity of endophytes

The dual culture technique was employed to test antagonistic effect of KL1 on the growth of *R. solani* and *F. oxysporum* (Zhou et al., 2011). An agar plug of 0.7-cm diameter from actively growing fungal mycelium was placed on the center of the PDA plate, and then KL1 were inoculated on the plate at 4 equidistance sites, 3 cm apart from the colony of *R. solani* or *F. oxysporum* in the center. Another plates were inoculated with the same

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size agar plug of fungal colony in the absence of KL1 as control. These plates were incubated at 24°C. And then the antagonistic effect of the KL1 on *R. solani* and *F. oxysporum* was observed.

1.4 Antifungal activity of KL1 supernatant and its thermal stability examination

Antifungal activity and thermal stability of cell free supernatant from KL1 were tested by measuring the ability to inhibit fungal growth of *R. solani* on PDA agar plates. KL1 was grown in 300 mL conical flasks containing 60 mL of No. 3S medium (Ohno et al., 1995) at 30°C and for 120 hr. Cell free supernatant was obtained by centrifugation at 16,000 ×*g* at 24°C for 10 min followed by filtration through 0.20-μm cellulose acetate filter. An agar piece (0.7-cm diameter) of *R. solani* was placed at the center of Petri dishes containing PDA. Each of the 200 μL, 150 μL, 100 μL, 50 μL of cell free supernatant was put into a penicillin-cup placed on the PDA, respectively. Thermal stability of the cell free supernatant was examined as follows: 300 μL of cell free supernatant was heat treated in a dry heat block (Dry Thermo Bath MG-2200 Eyela, Tokyo, Japan) for 15 min at 50°C, 55°C, 60°C, 65°C, 70°C or 90°C. After the heat treatment, each supernatant was tested for the remaining antifungal activity.

1.5 Antifungal activity by PDA medium containing 5%–15% KL1 supernatant

KL1 supernatant was sterilized by filtration through 0.20-μm membrane filter (DISMIC-25cs Cellulose Acetate 0.20-μm, Advantec, Tokyo, Japan) and added to sterilized PDA (5%–15%, *V*/*V*) of final concentration) (Coda et al., 2011). After mixing of the samples, aliquots of 10 mL were poured into petri plates (50-mm diameter). Control plates contained PDA alone. The assay was carried out by placing 0.7-cm-diameter plugs of growing *R. solani* onto the center of petri dishes containing the culture medium. Plates were incubated aerobically at 24°C. Mycelia growth inhibition was calculated as:

$$I = \frac{C - T}{C} \times 100 \quad (1)$$

where, I (%) is the mycelia growth inhibition, C (mm) is the mycelia diameter in control, and T (mm) is the mycelia diameter in PDA containing KL1 supernatant (Forchetti et al., 2007).

1.6 Extraction and HPLC analysis of iturin A and surfactin

For extraction of iturin A and surfactin, culture fluid was centrifuged at 16,000 ×*g* for 10 min. Then 500 μL of the supernatant was mixed with 500 μL of extraction buffer (CH_3CN:10 mmol/L ammonium acetate solution of 35:65 (*V*/*V*), and the mixture was vortexed for 10 min. The mixture was filtered through a 0.20-μm pore size polytetrafluoroethylene (PTFE) membrane (DISMIC-13JP, Advantec, Tokyo, Japan), and then injected into a high performance liquid chromatography (HPLC). HPLC analysis was performed using a JASCO LC-2000 series HPLC system (JASCO, Tokyo, Japan) equipped with a Chromolith Performance RP18e column at a flow rate of 2.0 mL/min. For separation, 10 mmol/L ammonium acetate aqueous solution and acetonitrile were used as mobile phase. The elution profile comprised an initial isocratic phase of 35% (iturin) or 45% (surfactin) acetonitrile for 10 min. Lipopeptide were detected by absorbance at 205 nm. Purified iturin A purchased from Sigma-Aldrich Co., USA and purified surfactin were used as standard samples for the HPLC.

1.7 Identification of antagonistic endophyte

Bacterial 16S rRNA genes were PCR-amplified with primers 357FWD-Bam (5′-GGGGATCCTCCTACGGGAGGCAGCAG-3′) and 1100RV-Bam (5′-CCGGATCCGGGTTGCGCTCGTTG-3′). The sequences of the amplified product were compared by BLAST search and identification was done based on similarity up to species level.

2 Results and discussion

To make sure the bacteria were isolated from the inside of the leaves, the surface sterilization was confirmed to be perfect by touching the surface of the leaves on TSA and PDA plates. One example is shown in **Fig. 1**. No colonies from the surface of the plant leaves were detected on the touching plates (**Fig. 1a, c**), but the colonies were emerged from inside of the leaves (**Fig. 1b, d**).

Six endophytic bacteria were isolated from 35 different plant leaves, and two were further screened and an isolate named KL1 having been isolated from the chestnut tree (*Castanea crenata*) leaves showed a strong antifungal activity as shown in **Fig. 2**.

Supernatant of the culture of KL1 also showed the strong inhibitory effect against the fungi, and the antifungal activity of supernatant was stable even after heating at 90°C for 15 min **(Fig. 3**).

In addition, growth of *R. solani* was inhibited on PDA medium containing supernatant of KL1 (**Fig. 4**). The inhibition effect was kept even after the 14 days and stability of the suppressive effect is a suitable characteristic as a biocontrol agent.

16S rRNA gene of KL1 was amplified using FWD357 and RV1100 primers. Based on partial 16S rRNA gene sequence, the isolated strain KL1 showed high sequence identity (99%) to *Bacillus* sp. by BLAST analysis. From these features, it is suggested that the compound extracted from KL1 supernatant might be cyclic lipopeptide iturin A. Therefore, qualitative analysis of iturin A and surfactin by HPLC were performed for the extract of the supernatant of KL1. As a result, clear iturin A homolog peaks, which