Endophytic Colonization of Balloon Flower by Antifungal Strain Bacillus sp. CY22

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Endophytic Bacillus sp. CY22 was previously isolated from the root interior of the balloon flower (Platycodon grandiflorum) (Cho et al., Biosci. Biotechnol. Biochem., 66, 1270–1275 (2002)). Three-month-old balloon flower seedlings were inoculated with 107 cfu/ml of strain CY22R3, a rifampicin-resistant strain of CY22, and external and internal root colonization was assessed 2 and 4 weeks later. After inoculation, large numbers of bacteria were observed on the root surface by scanning electron microscopy. More detailed studies using optical and transmission electron microscopy confirmed that Bacillus sp. CY22 was endophytically established within intercellular spaces, cortical cells, and aerenchymas of root. Also, Bacillus sp. CY22 showed antibiotic activities against several phytopathogens by producing the antibiotic iturin A. In the pot test, root rot of balloon flower seedlings caused by Rhizoctonia solani was suppressed when the Bacillus sp. CY22R3 was inoculated into the soil.

Key words: endophytic Bacillus sp. CY22; antibiotic iturin A; biological control

The root systems of plants offer different micro-habitats for bacterial growth. The bacteria are able to live in different tissues of healthy plants without causing symptoms of plant damage and give benefit to plants.13) These bacteria have been defined as endophytes by Kado.14) Since 1940 there have been numerous reports on indigenous endophytic bacteria in various plant tissues, including seeds and ovules,3) tubers,4) roots,5) stems and leaves,6) and fruits.7,8) So far, endophytic bacteria isolated from the internal tissue of healthy plants comprise over 129 species representing over 54 genera9–12) with Pseudomonas, Bacillus, Enterobacter, and Agrobacterium being the most commonly isolated bacterial genera. Early reports regarded endophytic bacteria as contaminants resulting from incomplete surface disinfection or latent pathogens.13,14) Since the 1990s, many studies have demonstrated that bacterial endophytes can improve plant growth and biological nitrogen fixation, and reduce disease symptoms caused by several plant pathogens.15–20) Recent research with endophytic bacteria has focused on their beneficial effects and ecology16,21) to better understand how bacterial endophytes interact with their host plants. In several cases, endophytic bacteria have been considered as potential biocontrol agents.15,16,18,19) In targeting fungal diseases, endophytic bacteria have shown obvious control in model systems of Fusarium oxysporum sp. vasinfectum15) and Rhizoctonia solani22) on cotton, Verticillium alboatrum and Rhizoctonia solani on potatoes,23) and Sclerotium rolfsii on beans.22) Among endophytic bacteria, Gram-negative bacteria, especially Pseudomonas strains, have been intensively investigated as biological control agents with regard to the production of antimicrobial metabolites24–27) The Gram-positive bacteria, like Bacillus sp., however, have been studied less intensively than Gram-negative bacteria. Since 1990, Bacillus sp. have been developed as fungal disease control agents, because Bacillus sp. are capable of producing antibiotics as secondary metabolites,28,29) as well as a variety of fungal cell-wall-degrading enzymes, such as cellulases, amylases, glucanases, etc.50–52) Many Bacillus strains produce small peptides with a long fatty moiety, the so-called lipopeptide antibiotics. The peptide portions of these compounds contain β-amino fatty acids with a n-configuration and are produced nonribosomally with templates of the multifunctional peptide synthetases.28,33,34

The balloon flower (Platycodon grandiflorum) is a widely cultivated vegetable and used as a remedy for asthma in East Asia. Especially, over-ten-years-old balloon flower root is one of the most cultivated

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medicinal crops, but 3 to 5-year-old balloon flower root is generally prone to disease, such as root rot caused by *Rhizoctonia solani*. Therefore, developing a biological control agent against balloon flower root rot is very important.

Endophytic *Bacillus* sp. CY22, which was isolated from the interior of balloon flower root, produced the antibiotic iturin A. Iturin A is widely known for its strong antifungal activity, and a valuable antifungal agent for its large spectrum and low toxicity. In this study, we described how to find whether *Bacillus* sp. CY22 could colonize balloon flower systemically, localize the colonization sites of *Bacillus* sp. CY22 in balloon flower roots, and assess whether *Bacillus* sp. CY22 would make an effective biological control agent by testing against the root rot of balloon flower caused by *R. solani*.

Materials and Methods

**Microorganisms and culture conditions.** *Bacillus* sp. CY22 was originally isolated from the interior of a balloon flower root (*Platycodon grandiflorum*) and strain CY22R3 was obtained by spontaneously mutagenesis. *Rhizoctonia solani* was kindly provided by the Laboratory of Phytopathology, Gyeongnam Agricultural Research and Extension Services, Chinju, Korea. The *Rhizoctonia solani* was maintained on potato dextrose agar (PDA, Difco). The strain CY22 was cultivated on tryptic soy agar (TSA, Difco) and the strain CY22R3 was cultivated on TSA medium containing 100 μg/ml of rifampicin. The *Rhizoctonia solani*, strain CY22 and strain CY22R3, were grown at 28°C.

**Inoculation of balloon flower with Bacillus sp. CY22R3.** Plants were prepared as described previously. The strain CY22R3 was grown in TSA broth supplemented with 100 μg/ml rifampicin until it reached an optical density of 0.6. The cells were then harvested by centrifugation, washed twice with 10 mM phosphate buffer, and resuspended in 10 mM phosphate buffer (10^7 cfu/ml). Plants were inoculated by pipetting 5 ml (10^7 cfu/ml) of strain CY22R3 suspensions. Plants inoculated with 5 ml of 10 mM phosphate buffer were used as controls. After inoculation, plants were grown in growth chamber (14 h light, 10 h dark cycle) at 22°C for 3 months.

**Enumeration of Bacillus sp. CY22R3 colonizing balloon flower roots.** Plants were sampled at various times, from 1 to 30 days after inoculation. Loosely attached bacteria were removed by washing roots with excess sterile water, and roots were then immersed in 10 mM phosphate buffer and vortexed for 30 s. The resulting solution was serially diluted and placed on TSA plates containing 100 μg/ml rifampicin. Bacterial colonies were then counted, and these counts were assumed to be those bacteria that were closely associated with the root surface. In another set, the roots were surface sterilized by immersion in 95% ethanol for 5 min followed by treatment with 1% sodium hypochlorite for 15 min. After three washes with sterile distilled water, surface-sterilized roots were removed approximately 0.5 cm from the margin of the root with sterile razor blades to remove external contamination. The roots were triturated by pestle in 10 mM phosphate buffer (pH 7.2) and plated on TSA plates as described above and enumerated.

**Microscopic study of colonization by Bacillus sp. CY22R3.** At least three seedlings from three independent inoculations were collected. The roots were cut into small pieces and fixed in 4% glutaraldehyde. The fixed samples were prepared following a protocol described previously. The root tissues were observed optically (ZEISS, model Axiostar) after Gram staining, scanning electron microscope (Philips, model XL 30 SF) and transmission electron microscope (Jeol, model JEM 1010). Uninoculated plants were used as control.

**Isolation and mass spectrometry of an antifungal substance.** Isolation of antifungal substance produced by strain CY22 was done essentially as described by Nakano et al. The antifungal substance fraction was separated by thin-layer chromatography (TLC) by using silica gel 60-plates (Merck). A mixture of chloroform/methanol/water 62:25:4 (v/v/v) was used as the eluent system. The various spots were made visible by charring after spraying with concentrated H_2SO_4. Finally, the fraction was purified by analytical reversed-phase high-pressure liquid chromatography (RP-HPLC; ODS Hypersil 5 μm C_18 column, 200 × 4.6 mm, Hewlett Packard). The following conditions were used: flow rate of 2.5 ml/min, acetonitrile/water 1:1 (v/v) as the mobile phase. The purified lipopeptide was dissolved in dimethyl sulfoxide and a plate assay was done out as described by Ryu et al. The antifungal substance was analyzed by using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF mass). Samples were dissolved in dimethyl sulfoxide/glycerol, and positive ions were detected. MALDI-TOF mass spectra were recorded on a Bruker (Bruker Daltonik, Bremen) Reflex MALDI-TOF instrument with a 337-nm nitrogen laser for desorption and ionization. A saturated solution of α-cyano-4-hydroxycinnamic acid in 70% acetonitrile:0.1% trifluoroacetic acid (v/v) was used as the matrix. Ions were accelerated with a voltage of 20-kV. The positive-ion and reflector mode was used.

**Biocost control activity of balloon flower inoculated with Bacillus sp. CY22R3.** The soil used in this study was a low humid andosol taken from a field at the
Gyeongnam Agricultural Research and Extension Services, Chinju, Korea and prepared following previously described methods. The twenty milliliters of suspension culture \((2 \times 10^8 \text{cells/ml})\) of CY22R3 was inoculated with 150 g of soil in a pot and soils were then inoculated with 1.5 g of 5 mm plugs taken from a PDA medium culture of \(R.\ solani\). The soil inoculated only with CY22R3 and \(R.\ solani\) was used respectively as the control. The three inoculations into soil were done simultaneously 3 days before transferring the balloon flower seedlings to the pot. For each treatment, six pots were prepared and experiments were repeated at least three times.

**Results**

**Evaluation of Bacillus sp. CY22R3 in external and internal root tissue**

To study the colonization of balloon flower by *Bacillus* sp. CY22, strain CY22 was mutated with rifampicin resistance and inoculated onto balloon flower seedlings under aseptic conditions. The CY22R3, rifampicin resistant mutant of CY22 was reisolated from the root surfaces of the inoculated plants. Strains CY22R3 had established large rhizosphere populations (i.e., \(>5 \times 10^7\) cells/g fresh root tissue) 2 weeks after inoculation of balloon flower seedlings (Fig. 1). The rhizosphere population of CY22R3 declined slightly when colonization was assessed 4 weeks after inoculation, but populations remained greater than \(5 \times 10^6\) cfu/g fresh root tissue. The strain CY22R3 could be reisolated from surface-sterilized roots of the inoculated seedlings, which indicates internal colonization within the plants. Strain CY22R3 showed a population size of \(5 \times 10^7\) cfu/g fresh root tissue (Fig. 1, 2 weeks after inoculation) and decreased gradually 4 weeks after inoculation.

No bacteria could be isolated from the control plants. Throughout the study, both control and inoculated plants did not show any visible disease symptoms.

**Root colonization of balloon flower by Bacillus sp. CY22R3**

A SEM survey of balloon flower roots at 4 days after inoculation with CY22R3 showed that high populations of CY22R3 cells were located on the root surface of the balloon flower (Fig. 2A and 2B). Light and transmission electron microscopy of inoculated plants showed that in addition to the surface colonization (Fig. 4A and 4B), there was also considerable internal colonization of the roots (Fig. 4C–4F). Transverse section of inoculated roots revealed the presence of bacteria within many cortical intercellular spaces next to the stele and within the aerenchyma (Fig. 4C–4F), although there was no evidence that the bacteria had penetrated the endodermis to colonize the root vascular system. The bacteria were observed on the root surface (RS), in grooves between epidermal cells (EC), and in intercellular spaces (IS) between inner root cortexes (RC) (Fig. 4A–4E). Also, a number of *Bacillus* sp. CY22R3 cells were located in intercellular spaces of the root cortex close to the
conducting elements (Fig. 4D). The bacteria were not found within the xylem of roots (Fig. 4F).

**Antifungal substance produced by Bacillus sp. CY22**

Antifungal substance production in strain CY22 was investigated using chromatographic procedures and FAB mass spectrometry and MALDI-TOF mass spectrometry. Acid precipitates of the culture supernatants were extracted with methanol and detected by thin-layer chromatography (TLC) on silica gel 60-plates. Two prominent spots became visible by charring with H$_2$SO$_4$ and $R_f$ values of these two spots were 0.38 and 0.68. The spot of 0.38 $R_f$ value, which showed antifungal activity against Rhizoctonia solani, Phytophthora ultimum, and Fusarium oxysporum (data not shown) was purified by HPLC for further analyses. The FAB mass spectrum of the purified compound displayed three [M + H]$^+$ peaks at $m/z = 1043.4$, 1057.4, and 1071.4 (data not shown). Comparison with a mass database indicated that peaks at $m/z$ 1043.4, 1057.4, and 1071.4 corresponded to the known lipopeptide iturin A. The MALDI-TOF mass spectrum of the purified compound is shown in Fig. 5. Three [M + H]$^+$ peaks occurred at $m/z$ 1043.39, 1057.42, and 1071.42. These peaks were shifted to $m/z$ 14 lower indicating isomers of iturin A. One series of ion was found at $m/z$ 1065.42 and 1079.42 indicating sodium adducts (Fig. 5).

**Biocontrol potential of Bacillus sp. CY22**

To investigate the suppressive effects of strain CY22 on the root rot of balloon flower caused by *R. solani*, we did pot experiments using strain CY22R3, a rifampicin-resistant mutant of strain CY22, and 3-months-old balloon flower seedlings. Among the three treatments, all balloon flower seedlings inoculated with only strain CY22R3 grew normally and no disease appeared. In the pots inoculated with only *R. solani*, 86% of balloon flower seedlings showed disease symptoms. However, when both strain CY22R3 and *R. solani* were inoculated into soil, the percentage of root rot diseased balloon flower seedlings decreased to 60% (Table 1).

**Discussion**

*Bacillus* sp. CY22 was able to colonize the intercellular space of balloon flower root without harm to the balloon flower and produced a potent antifungal lipopeptide such as iturin. To study plant colonization by the isolated bacterium, *Bacillus* sp. CY22 was mutated to become spontaneously rifampicin resistant and was inoculated onto balloon flower seedlings under aseptic conditions. The rifampicin resistant strain, designated CY22R3, was reisolated...
Fig. 5. MALDI-TOF Mass Spectrum of the Lipopeptide Iturin Fraction of Bacillus sp. CY22.

The cyclic lipopeptide appeared as a complex mixture of several isoforms that show variations in the length of their ω-amino fatty acid moiety (n = 10 to 12 CH₂ groups). The [M + H]⁺ peaks at m/z 1043.39, 1057.42 and 1071.42 were accompanied by the corresponding [M + Na]⁺ peaks (m/z 1065.42 and 1079.42).

Table 1. Effects of Bacillus sp. CY22 on the Suppression of Root Rot of Balloon Flower Caused by R. solani in Pot

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<tr>
<th>Treatment</th>
<th>Rhizoctonia solani</th>
<th>Root rot diseased plants (%)</th>
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<tr>
<td>+</td>
<td>Bacillus sp. CY22R3</td>
<td>85 ± 5</td>
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<tr>
<td>+</td>
<td>+</td>
<td>60 ± 5</td>
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<td>+</td>
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from surface-sterilized roots of the inoculated seedlings, indicating internal colonization within the plant roots. Therefore, to prove that Bacillus sp. CY22 is genuinely endophytic in the balloon flower, the inoculated balloon flower roots were fixed with glutaldehyde, embedded in resin, and sectioned for optical and electron microscopy. Inside the roots, bacteria were mainly localized within the epidermal cells and intercellular spaces.39,40 These apoplastic locations seem to be the preferred sites of the few endophytic bacteria so far examined in plants. For example, Quadt-Hallmann et al.41 have shown dense colonies of Enterobacter asburiae in the intracellular spaces of the inner cortex of cotton roots, and Gyaneshwar et al.17 have presented micrographs showing Serratia marcescens extensively colonizing the intercellular spaces and aerenchyma of rice roots. In the present study, the bacteria were not found within the xylem of the roots. This is similar to work of Gyaneshwar et al. on Serratia marcescens in rice. But this contrasts with previous works on Azoarcus and seropedicae that had shown colonization of root xylem vessels, albeit at a relatively low frequency.

Although endophytes have been identified in various plants and postulated to play an important role in sustainable crop production,42 the mechanisms of endophytic establishment are not well known. It has been suggested that the bacteria could enter through the fissures created by the emergence of lateral roots or could actively dissolve the cell wall components to gain entry.10,43 We found that Bacillus sp. CY22 secretes cellulase and xylanase (data not shown). However, release of plant cell wall degrading enzymes by endophytic bacteria is still controversial since this would confer plant pathogenicity.10 Since the 1990s, many studies with endophytes have described the biocontrol potential of endophytic bacteria. In this study, we identified that endophytic Bacillus sp. CY22 isolated from balloon flower root produced iturin A with antifungal activity against Rhizoctonia solani, Phytium ultimum, and Fusarium oxysporum.

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References


