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Screening of plant growth-promoting rhizobacteria as elicitor of systemic resistance against gray leaf spot disease in pepper

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ABSTRACT

This study investigated the effects of plant growth-promoting rhizobacteria (PGPR) isolated from Dokdo Island for growth promotion of pepper and biological control activity against a gray leaf spot disease pathogen, *Stemphylium lycopersici*. Screening of PGPR was carried out in the rhizosphere of wild plant *Elymus tsukushiensis* from Dokdo. Rhizobacterial isolates were partially identified based on analysis of 16S rDNA sequences. Phylogenetic analysis was performed using sequences of bacterial isolates for comparative purposes. To select PGPR, all bacterial isolates were tested for phosphate solubilization, production of indole-acetic acid (IAA), and siderophores. Isolates positive for all three characteristics were selected and tested for growth promotion of pepper as well as potential biological control of *S. lycopercisi*. All selected isolates were able to enhance plant growth with *Kluyvera cryocrescens* KUDC1771 showing the highest plant growth promoting activity. Among selected isolates, four significantly decreased gray leaf spot disease severity with *Brevibacterium iodinum* KUDC1716 providing the highest disease suppression. Moreover, KUDC1716 enhanced expression of *pathogenesis-related* (*PR*) protein genes including *CaPR4* and *CaChi2* in the absence of pathogen. These results suggest that *B. iodinum* induce defense response against *S. lycopersici* and can be used as a potential agent for biological control.

1. Introduction

Dokdo is a volcanic island located in the northeastern part of Ulleungdo, which is the easternmost region of South Korea. This region contains approximately 89 small islands and rocks, including Dongdo and Seodo Islands. Dokdo Island has disadvantageous conditions for plant growth such as drought, strong winds, steep inclinations, soil salinity, high uric acid concentration, and lack of organic nutritive elements. Despite the harsh environment for plant survival, Dokdo contains a healthy plant flora, including 61 species (Cultural Heritage Administration of Korea, 2009a,b). Among these plants, *Elymus tsukushiensis*, one of the dominant plant communities of Dokdo, displays healthy plant-microbe interactions with plant growth-promoting rhizobacteria (PGPR) (Ham et al., 2009; Jeon et al., 2009).

PGPR are free-living bacteria that colonize the rhizosphere with beneficial effects on plant health. PGPR can stimulate plant growth, protect plants from pathogen infection, and reduce the effects of abiotic stresses, leading to increased crop yields (Babalola, 2010). Enhanced plant growth by PGPR can be attributed to the production of plant growth-promoting hormones, including auxin, gibberellin, and cytokinin, as well as the increased availability of limited nutrients such as phosphorous, iron, nitrogen, vitamins, and amino acids (Lugtenberg and Kamilova, 2009; Ryu et al., 2003). Among phytohormones, IAA is the primary hormone produced by PGPR and is known as the major auxin in plants. Further, IAA is widespread in the whole body of plants, especially in meristematic tissue and growing regions, including germinating seeds; tip of the stem or root and in terminal buds. In addition, IAA elicits various important physiological processes such as division of vascular bundles, tropistic responses, and development of lateral buds, flower, and fruit. Phosphate solubilization by PGPR is one of the most important factors in plant growth promotion. Phosphorous is considered one of the most essential elements for plant growth and development as it participates in energy metabolism and is an important component of nucleic acids such as ATP and ADP in plants (Taiz and Zeiger, 2003). In fact, large amounts of phosphate are present in farmland due to chemical fertilizers (Belimov et al., 1995; Vessey, 2003). However, phosphates in soil are rapidly converted to insoluble form upon combining with iron (Fe^{3+}), aluminum (Al^{3+}), or calcium ion (Ca^{3+}) (Paul and Clark, 1989), resulting in soluble phosphorous for plant use. In addition, absorption of limited mineral by PGPR plays an important role in plant growth. Especially, siderophores produced are secondary metabolites in soil with high affinity iron chelating compound secreted by PGPR. Even though iron is an essential nutrient

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of plants, it is relatively insoluble in soil (Vessey, 2003). Therefore, siderophore produced by PGPR play an important role to supply iron in plant.

PGPR possess various direct and indirect mechanisms that help to suppress phytopathogens (Kloepper, 1993; Lugtenberg and Kamilova, 2009; Babalola, 2010). Direct mechanisms involve production of antibiotics, siderophores, and lytic enzymes such as glucanase and chitinase (Kloepper et al., 1988; Slininger. et al., 2004; Kim and Kim , 2008). On the other hand, indirect mechanisms include induction of local or systemic resistance against wide array of viral, bacterial, and fungal pathogens (Ham et al., 2009; Phi et al., 2010; Son et al., 2012). PGPR also possess various inducible mechanisms for defense against biotic stress. These mechanisms can be triggered by chemical agents in addition to avirulent, incompatible, or virulent pathogens. Most commonly, induced resistance in plants is systemic as defense capacity is enhanced in both non-infected as well as primary infected tissues. Thus, induction of pathogen resistance in plants is a state of enhanced defensive capacity resulting from proper stimulation by diverse agents such as rhizobacteria (Van Loon et al., 1998). Generally, there are two kinds of defense mechanisms, systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Van Loon et al., 1998). SAR is activated by pathogens, resulting in limited infection such as hypersensitive reactions (Choi and Hwang, 2011). On the other hand, ISR confers enhanced defensive capacity to whole plants not only at sites of rhizobacteria colonization, resulting in a reduced rate of disease development, fewer diseased plants, and lower disease severity (Zehnder et al., 2001). Both SAR and ISR are regulated by distinct signaling pathways. Specifically, SAR involves accumulation of salicylic acid and expression of *pathogenesis-related* (*PR*) protein genes, whereas ISR is not always accompanied by activation of *PR* protein expression (Van Loon, 2007). In addition, 1,3-glucanase and chitinase are among those *PR* genes expressed for hydrolysis of fungal cell walls. Production of *PR* proteins is important as they enhance overall resistance in whole plants against diverse pathogens (Adrienne and Barbara, 2006). ISR is further potentiated by PGPR and relies on pathways regulated by jasmonate and ethylene (Bakker et al., 2003).

Gray leaf spot disease in pepper seedlings was first reported by Sinclair et al. (1958) and Blazquez (1969). This disease occurs most often in mountainous areas exposed low temperatures at night (Cho et al., 2001). The main pathogens of gray leaf spot disease are *Stemphylium lycopersici* and *S. solani*, and symptoms include spots no greater than 3 cm in diameter spots with dry, white, and sunken centers. The disease develops by interrupting photosynthesis, resulting in numerous tiny brown to yellowish leaf spots and severe defoliation (Kim et al., 2004). Although chemical fungicides have been used, no formal chemical or biological agent has been shown to be effective. Further, chemical agents have toxic effects on human and animal health by acting as ecocides, resulting in secondary pollution of the environment (Lee, 1997). Further, current trends encourage the production of organic vegetables and foods for improved personal well-being. Therefore, use of PGPR as biological agents could be an effective environment-friendly alternative in the agriculture industry (Shen et al., 2005; Mena-violante and Olalde-Portugal, 2007).

Almost all previous PGPR screenings were restrictively carried out only under farm-field conditions (Joshi and Bhatt, 2011; Farina et al., 2012). However, studies on the identification of PGPR in the rhizosphere of wild plants have not been performed. The present study has been carried out to explore the microbial diversity of PGPR associated with the rhizosphere of *E. tsukushiensis* from Dokdo Island as well as screen bacterial isolates for their plant growth-promoting activities and induction of systemic resistance against gray leaf spot disease.

2. Materials and methods

2.1. Isolation of microorganisms

All isolates were collected from the rhizosphere of wild *E. tsukushiensis* from Dokdo Island. To screen rhizobacteria, 1 g of rhizosphere soil was mixed with 9 ml of sterilized saline (0.85% NaCl), followed by shaking at 50 rpm for 20 min. Soil suspension was gradually diluted and spread on 1/10 tryptic soy agar (TSB, Difco, USA) media, after which plates were incubated at 30 °C for 2 days. Bacterial isolates were characterized based their shape and color and then isolated as a pure culture. For long-term preservation, all bacterial isolates were stored in 15% glycerol stocks at −70 °C until use.

2.2. Cultivation of plants

Pepper (*Capsicum annuum* L.) seeds (Manita, Nongwoobio, Korea) were surface-sterilized with 1.2% hypochlorite for 30 min, washed 30 min with tap water, and dried at room temperature. Seeds were planted in a 50-hole plastic tray pot (7 cm in diameter) filled with sterilized commercial soil. Pepper plants were grown in a plant growth room under a 12-h light/12-h dark cycle at 25 °C with 50% humidity. Pepper plants in early 4 leaf stage were used for plant growth promotion assay and induction of resistance against gray leaf spot disease.

2.3. Incubation of pathogen

S. lycopersici, the causal pathogen of gray leaf spot disease, inoculum was prepared as described by Kim et al. (2004). Pathogens were grown on a V-8 juice agar plate [200 ml of V-8 Juice (Campbell, USA), 3 g of $CaCO_3$, 20 g of agar, and 800 ml of distilled water], followed by incubation in a growth chamber under 12 h of fluorescent light at 20 °C and 12 h of dark at 15 °C to induce spore formation. To isolate conidial spores for pepper infection, 7 ml of sterile distilled water (SDW) was poured into each plate, after which spores were gently rubbed off from the mycelial surface using a plastic loop. Spore suspension was filtered through three-layer cheese cloth and adjusted to 5.0×10^3 spores/ml for induction of systemic resistance assay. For long-term storage, spores were maintained in 20% sterile glycerol at −70 °C.

2.4. Partial identification of bacterial isolates

Bacterial isolates were partially identified by analysis of 16S rDNA sequences. Genomic DNA from isolates was extracted using a Wizard Genomic DNA Purification Kit (Promega, USA). The 16S rDNA genes were amplified by PCR using the universal sequencing primer 518F as the forward primer (5′-CCA GCA GCC GCG GTA ATA CG-3′) and 800R as the reverse primer (5′-TAC CAG GGT ATC TAA TCC-3′) at the Genomic Division, Macrogen Inc., Korea using BigDye (R) Terminator v3.1 Cycle Sequencing Kits (Applied Biosystem, USA). The results of the 16S rDNA sequence analysis were compared with registered sequences in the GenBank database using NCBI Blast server (http://www.ncbi.nlm.nih.gov). The 16S rDNA sequences were deposited in the GenBank database of NCBI.

2.5. Plant growth-promoting characteristics

To identify the beneficial effects of PGPR in plants and select specific PGPR for plant growth promotion and induction of systemic resistance in pepper, production of indole-3-acetic acid (IAA) and siderophores was measured, and phosphate solubilization assays were conducted using all isolates.