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Antagonistic endophytic bacteria associated with nodules of soybean (*Glycine max* L.) and plant growth-promoting properties

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ABSTRACT

A total of 276 endophytic bacteria were isolated from the root nodules of soybean (*Glycine max* L.) grown in 14 sites in Henan Province, China. The inhibitory activity of these bacteria against pathogenic fungus *Phytophthora sojae* 01 was screened *in vitro*. Six strains with more than 63% inhibitory activities were further characterized through optical epifluorescence microscopic observation, sequencing, and phylogenetic analysis of 16S rRNA gene, potential plant growth-promoting properties analysis, and plant inoculation assay. On the basis of the phylogeny of 16S rRNA genes, the six endophytic antagonists were identified as belonging to five genera: *Enterobacter*, *Acinetobacter*, *Pseudomonas*, *Ochrobactrum*, and *Bacillus*. The strain *Acinetobacter calcoaceticus* DD161 had the strongest inhibitory activity (71.14%) against the *P. sojae* 01, which caused morphological abnormal changes of fungal mycelia; such changes include fracture, lysis, formation of a protoplast ball at the end of hyphae, and split ends. Except for *Ochrobactrum haematophilum* DD234, other antagonistic strains showed the capacity to produce siderophore, indole acetic acid, and nitrogen fixation activity. Regression analysis suggested a significant positive correlation between siderophore production and inhibition ratio against *P. sojae* 01. This study demonstrated that nodule endophytic bacteria are important resources for searching for inhibitors specific to the fungi and for promoting effects for soybean seedlings.

Introduction

The root nodules of legume plants are symbiotic organs induced by soil bacteria known as rhizobia. As part of the root system, root nodules harbor symbiotic bacteria and many endophytes, including *Agrobacterium tumefacien*,[1] *A. rhizogenes*,[2] *Phyllobacterium*, *Stenotrophomonas*, *Enterobacteriaceae*,[3] *Bacillus* species,[4] *Bacillus*, *Bordetella*, *Curtobacterium*, and *Pantoea*.[5] Aside from their diversity, which

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has been studied extensively, the effect of nodule endophytes on host legumes was revealed. The nodule endophytic *Agrobacterium* strains specifically inhibit the nodulation of *Rhizobium gallicum* on the common bean (*Phaseolus vulgaris* L.)[6] or facilitate the unspecific nodulation of *Sinorhizobium meliloti* on woody legumes.[7] Some nodule endophytes that belong to *Bacillus*, *Bordetella*, *Curtobacterium*, or *A. rhizogenes* could promote the growth or nodulation of red clover.[5] These phenomena are similar to that of endophytes isolated from other parts of plants and could benefit host plants by producing phytohormones, 1-aminocyclopropane-1-carboxylase (ACC) deaminase, and antibiotic compounds, as well as by fixing nitrogen, solubilizing phosphate, or suppressing phytopathogens through the competence of invasion sites.[8–11] Owing to the above mentioned advantages, endophytes are considered novel resources in the biocontrol of plant diseases and in the promotion of plant growth.[12–14]

As a major legume crop, soybean (*Glycine max* L.) plays an important role in sustainable agriculture and in the economy of many countries. Soybean has a great nitrogen-fixing ability due to its symbiosis with rhizobia in root nodules. The presence of *Bradyrhizobium japonicum*, *B. liaoningense*, *B. yuanmingense*, *B. elkanii*,[15,16] *B. huanghuaihaiense*,[17] *B. daqingense*,[18] *B. pachyrhizi*, *B. iriomotense*, *B. canariense*,[19] *Sinorhizobium fredii*, and *S. sojae*[20] has been reported in China, which is the center of origin of soybean.[21,22] Similar to other plants, endophytic bacteria have been isolated from different parts of soybean,[19,23–26] and some of these parts showed antagonistic and growth-promoting potential.[27–29] Diverse endophytic bacteria, including *Pantoea*, *Serratia*, *Acinetobacter*, *Bacillus*, *Agrobacterium*, and *Burkholderia*, have also been isolated from soybean nodules.[30] However, antagonistic endophytic bacteria within nodules of soybean for *P. sojae* in Henan Province have not been sufficiently studied.

On the basis of the above mentioned background knowledge and considering the nodule endophytes as a new bacteria resource with potential in biotechnology, we conducted this study (1) to screen antagonistic endophytic bacteria from soybean nodules against *P. sojae*; (2) to explore the potential plant-beneficial traits of endophytic bacteria; and (3) to assay the seedling growth response of soybean to the inoculation of endophytic bacteria.

Materials and methods

Collection of root nodules, soil samples, phytopathogenic fungus, and soybean seeds

Nodules from cultivated soybean were collected from July to August 2012, when the plants were blooming. Samples were obtained from fields of 14 sites subordinate to 9 districts of Henan Province, China (map available as Supplementary Fig. 1).[21,22] Three healthy root nodules with similar sizes were excised from the lateral roots of each plant. Soil debris was brushed away from the nodules, and the nodules were stored in sterile plastic bags at 4 °C until they were processed for isolation within 24 h.

In each site, soil cores were sampled at five locations with a depth of 15–20 cm and 5 cm away from the taproots, which were bulked and thoroughly mixed to form composite samples. Soil samples were stored in loosely tied plastic bags at 4 °C. Soil texture was defined according to the international institution triangle coordinate graph, and soil pH was determined as described in Zhao et al.[31]

A phytopathogenic fungus, *P. sojae* 01, was provided by the College of Life Sciences of Northwest A & F University in China and was incubated on potato dextrose agar plate (PDA: extract of 200 g potato, 20 g of glucose, 18 g of agar, 1 L of distilled water) at 30 °C for 3 days and maintained at 4 °C for temporary storage.

The seeds of soybean (*G. max* L.) cultivar Zhonghuang 13, which is the principle cultivar used in the sampling region, were bred by the Institute of Crop Sciences of the Chinese Academy of Agricultural Sciences.

Isolation and purification of soybean nodule endophytes

Bacteria were isolated from root nodules according to a standard method as described by Ma et al.[32] and Miller et al.[33] A single colony of the isolate was repeatedly streaked on the same medium and examined with a microscope. Pure cultures were preserved on plates at 4 °C for temporary storage or in sterile vials with 30% (v/v) glycerol for long-term storage at −80 °C. To confirm if the surface sterilization process was successful, several surface-sterilized nodules were rolled over nutrient agar (NA) plates and aliquots of water from final rinse solutions and then plated onto NA plates.[34] Plates without any contaminants were considered effectively surface-sterilized, and the corresponding plates were used for the isolation of endophytes.

Screening of antagonistic endophytic bacteria

The antifungal activity of endophytes against pathogenic fungus *P. sojae* 01 was detected by using the point inoculation method.[35] Spores of fungal cultures were inoculated on PDA plates, and a small block of agar with fungal mycelia cut with a sterile puncher (Ø = 4 mm) was placed in the center of a fresh plate. Tested strains were spot inoculated on the edge of PDA plates approximately 25 mm from the center. After incubation at 28 °C for 7 days, the inhibition zone was measured. Fungal mycelia that were cultivated without inoculation were included as control.[36] Experiments were performed in triplicate for each bacterial isolate.

Secondary screening of antifungal activity was performed similar to the primary screening method, but bacteria were spot inoculated as bacterial suspension ($OD_{600} \approx 1$). Antagonistic activities were evaluated by measuring inhibition zones between pathogens and tested bacteria.

Microscopic observation of phytopathogenic fungi mycelia

To determine the effect of endophytic bacteria on pathogenic fungus, treated and untreated pathogenic fungi were cultured for 2 days on PDA medium. The morphological changes of pathogenic fungus caused by endophytes were examined under an optical epifluorescence microscope (BX50 Olympus) at 200-fold magnification and compared with the structures of the control groups. The mycelium of each pathogenic

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