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Bacillus amyloliquefaciens SB14 from rhizosphere alleviates *Rhizoctonia* damping-off disease on sugar beet

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ABSTRACT

The use of biocontrol strains recently has become a popular alternative to conventional chemical treatments. A set of bacteria isolated from sugar beet rhizosphere and from roots and shoots of apple and walnut were evaluated for their potential to control sugar beet seedling damping-off caused by R. solani AG-4 and AG2-2.The results of in vitro assays concluded that three isolates, SB6, SB14, SB15, obtained from rhizosphere of sugar beet and five isolates, AP2, AP4, AP6, AP7, AP8, obtained from shoots and roots of apple were the most effective antagonists that inhibited the mycelial growth of both *R. solani* isolates. Combination of several biochemical tests and partial sequencing of 16S rRNA and *gyrB*genes revealed that eight efficient bacterial isolates could be assigned to the genus *Bacillus* and all could tolerate high temperatures and salt concentrations in their vegetative growth. The potential biocontrol activity of the eight bacterial antagonists were tested in greenhouse condition. The results indicated that four strains,*B. amyloliquefaciens* SB14, *B. pumilus* SB6,*B. siamensis* AP2 and *B. siamensis*AP8 exerted a significant influence on controlling of seedling damping-off and performed significantly better than others.However, the treatment of the seeds with bacteria was most effective when the isolate SB14 was used, which significantly controlled damping-off disease by 58% caused by *R. solani* AG-4 and by 52.5% caused by *R. solani* AG-2-2. This indicates that the use of beneficial bacterial native to the host plant may increase the success rate in screening biocontrols, because these microbes are likely to be better adapted to their host and its associated environmental conditions than are strains isolated from other plant species grown in different environmental conditions. We can infer from the results reported here that sugar beet plantsmay recruitbeneficial microbes to the rhizosphere to help them solve context-specific challenges.

1. Introduction

Very often plant pathogenic fungi are attacking plants and are a serious and constant hazard to agriculture and ecosystem sustainability. It has led to consequential economic losses, particularly over the past few decades as agricultural production has increased. To face these impediments, producers have become extremely dependent on chemical use. However, intensive use of these compounds has led to resistance development in the pathogens and caused severe negative environmental impacts. Furthermore, consumers strong demand for pesticide-free food, has led to a decline of chemical usage in the fields(Gerhardson, 2002) and an increase of using natural beneficial microorganisms (biopesticides), also termed as biocontrol, for more rational and safe disease management (Postma et al., 2003; Welbaum et al., 2004; Whipps 2001).

Numerous studies have reported the potential use of rhizosphere-associated bacteria in stimulating plant growth and protecting them against fungal pathogens (Hallmann et al., 1997; Lucy et al., 2004; Lugtenberg and Kamilova 2009; Somers et al., 2004; Sturz et al., 2000). Among rhizosphere-associated bacteria, several strains belonging to the Gram-positive genus *Bacillus* were reported effective for the biocontrol of multiple plant diseases caused by soil-borne plant pathogens (Erlacher et al., 2014; Huang et al., 2012; Rajendran and Samiyappan 2008; Yu et al., 2002). *Bacillus* species are attractive for use in farming systems because of their ability to form spores resistant to UV light, heat and dryness which allows them to resist adverse environmental conditions, and permits easy formulation for commercial purposes (Piggot and Hilbert 2004; Tiago et al., 2004).

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The discrepancy in performance of beneficial bacteria such as *Bacillus* spp. under field conditions and variations in results from laboratory to field are more attributed due to various abiotic stresses that prevail under field conditions for biocontrol agents to establish and to show their desired effects. Many abiotic soil factors, such as pH, temperature, salt concentration, moisture, texture, and inorganic and organic constituents, influence on bacteria growth and consequently influence the specific activities occurring during antagonistic growth, such as antibiotic production, induction of host defence responses, etc. (Landa et al., 2004; Gopalakrishnan et al., 2012; Ownley et al., 2003). Such problems can be overcomed by screening programs for efficient stress tolerant biocontrol agents for effective deployment of them to draw one or more beneficial effects.

Soil-borne plant pathogens like *Rhizoctoniasolani*Kühn [teleomorph: *Thanatephoruscucumeris* (Frank) Donk] are difficult to be controlled(González García et al., 2006). This widespread fungus is responsible for serious damage to many important agricultural and horticultural crops as well as trees (Sneh et al., 1996). It is the most important and consistently occurring pathogen causing seedling root and crown rot diseases in sugar beet (*Beta vulgaris* subsp. *vulgaris*). Seedling damping-off caused by *R. solani* can seriously affect stand establishment of sugar beet, resulting in substantial economically losses (Whitney and Duffus, 1986). This pathogen is characterized by a high aggressiveness against sugar beet, a high tolerance against low and high temperatures, and a low sensitivity against fungicides (Büttner et al., 2003). Several *R. solani*anastomosis groups (AGs) including, AG-1, −2-1, −2-2, −3, −4 and −5 can cause damping-off of sugar beet.Of these mentioned earlier, AG-4 and −2-2 are the most predominant (Windeis and Nabben, 1989). AG 2-2 causes *Rhizoctonia* root and crown rot in sugar beet which is a destructive disease in the main growing areas (Buhre et al., 2009). Root yield and sugar content of plants infected by this disease are often reduced by 50% or more. Beet storage and processing quality are affected, resulting in difficulties of processing beet in the sugar factory (Büttner et al., 2004).

The use of chemical fungicides as seed treatment is the most common strategy to prevent this pathogen (Windels and Brantner, 2005). However, due to rising environmental contamination and appearance of resistant races of the pathogen, alternative and ecologically friendly regime to control damping-off on sugar beet comprises naturally occurring antagonists. Damping-off diseases decrement with antagonistic micro-organisms can be relatively easy, compared with other soil-borne diseases. The pathogen infects seed and seedlings, thus the antagonist needs to be introduced at seeds, and protection is necessary for only a few days or weeks thereafter (Georgakopoulos et al., 2002).

There are documents on bacterial suppression of *R. solani* on different plants (Adesina et al., 2009; Faltin et al., 2004; Grosch et al., 2005). Few attempts have been made to explore the possibility of bacterial biocontrol agents for the management of diseases of sugar beet caused by *R. solani*(Jorjani et al., 2012; Lovic et al., 1993; Moussa, 2002; Nielsen et al., 1998; Zachow et al., 2010). So far, there is a lack of comprehensive research on the effectiveness of sugar beet seed treatment using beneficial bacteria on protection of sugar beet against damping-off diseases. Here, we isolate the bacteria from sugar beet rhizosphere and from roots and shoots of apple and walnut. Then, we have initiated screening of antifungal activities of bacterial isolates against *R. solani* AG-2-2 and −4 isolates in vitro and thenstarted to phenotypically demonstrate the biocontrol activity of selected bacteriaagainst *R. solani*isolateson sugar beet as seed treatment. Furthermore, potential bacterial antagonists were identified using biochemical characteristics and partial sequencing of 16S rRNA and *gyrB* genes.

2. Material and methods

2.1. Sugar beet seeds and microorganisms

Seeds of sugar beet cv. Shirin, a *R. solani*-susceptible cultivar, and *R. solani* isolates have been originally isolated from naturally infected sugar beet plants which were provided by SugarBeet Seed Research Institute, Karaj, Iran. Bacterial and fungal isolates were maintained on nutrient agar (NA; Merck, Germany) and potato dextrose agar (PDA; Merck, Germany) slants at 4 °C, respectively and subcultured every 3-month intervals. For long-term storage, fungal isolates were conserved by culturing each isolate on sterile barley grains and storing at 4 °C.Bacterial isolates were stored at −80 in 30% sterile glycerol to preserve them for long period of times.

2.2. Isolation of bacteria and growth condition

A total of five different soil samples were collected from various sugar beet rhizosphere in Iran during 2011 and 2012. Approximately, 1 g of the soil samples were dissolved in 10 mL of sterile distilled water and shacked for 20 min on a rotary shaker at 250 rpm. Serial dilution was prepared and 20 μL of 10^{-5} till 10^{-8} was streaked on nutrient agar (NA; Merck, Germany) medium.Four shoot and root samples from symptom less apple trees and one shoot sample from walnut tree were collected from Azarbaijangharbi province.Canker symptoms were seen in the sampled orchards. Samples were placed individually in plastic bags and brought to laboratory for isolation of bacteria. Root and shoot samples were first sterilized by surface disinfection, by washingin running tap water, followed by a 70% ethanol wash for 1 min, a sodium hypochlorite solution (4%) wash for 2 min, ethanol for 30 s,and five serial rinses in sterilized distilled water. After disinfection, roots and shoots were sliced with a sterile scalpel and placed into NA medium. After 3 days of incubation at 27 °C, the obtained bacterial colonies were purified on NA plate three times and stored in 30% glycerol at −80 °C.

2.3. 3 R. solani*inoculum preparation and pathogenicity test*

Pathogenicity of ten *R. solani* isolates belonging to AG-2-2 and AG-4 (Table 1) were assessed according to Grisham and Anderson (1983). The inoculum for *R. solani* isolates were prepared by placing 200 g of corn seeds that were soaked in distilled water overnight into 500 mL Erlenmeyer flasks, and autoclaved at 121 °C for 20 min on two successive days. The substrate was inoculated with ten agar plugs (5 mm diameter) from the periphery of a 3-days-old culture of *R. solani* 124 and/or *R. solani* 133 isolates grown on PDA. The flasks were incubated for six weeks at 25 °C in dark with occasional shake to ensure uniformity of growth. Colonized corn seeds that had been similarly autoclaved served as control. Soil mix consisting of; soil, perlite and peat moss (3:1:1) was autoclaved for one hour on each of two consecutive days. Soil was mixed with the inoculum consisting of *R. solani*-infested corn seeds (8 g colonized and blended-corn seeds per kg air-dried soil) and pots were filled by inoculated soil. Seeds were initially surface-sterilized with 1% sodium hypochlorite for 2 min and then washed several times with distilled water. Forty sugar beet seeds were planted in each pot. Pots were incubated in the greenhouse under the following conditions: 25 °C, 40% humidity, 16 h light and 8 h dark. Experimental design for all treatments was randomized, complete block with four replications. Each experiment has been conducted twice. After 14 days of planting seeds, we recorded the number of healthy seedlings.