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Biocontrol of *Rhizoctonia solani* damping-off and promotion of tomato plant growth by endophytic actinomycetes isolated from native plants of Algerian Sahara☆

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ABSTRACT

Thirty-four endophytic actinomycetes were isolated from the roots of native plants of the Algerian Sahara. Morphological and chemical studies showed that twenty-nine isolates belonged to the *Streptomyces* genus and five were non-*Streptomyces*. All isolates were screened for their *in vitro* antifungal activity against *Rhizoctonia solani*. The six that had the greatest pathogen inhibitory capacities were subsequently tested for their *in vivo* biocontrol potential on *R. solani* damping-off in sterilized and non-sterilized soils, and for their plant-growth promoting activities on tomato seedlings. In both soils, coating tomato seeds with antagonistic isolates significantly reduced ($P < 0.05$) the severity of damping-off of tomato seedlings. Among the isolates tested, the strains CA-2 and AA-2 exhibited the same disease incidence reduction as thioperoxydicarbonic diamide, tetramethylthiram (TMTD) and no significant differences ($P < 0.05$) were observed. Furthermore, they resulted in a significant increase in the seedling fresh weight, the seedling length and the root length of the seed-treated seedlings compared to the control. The taxonomic position based on 16S rDNA sequence analysis and phylogenetic studies indicated that the strains CA-2 and AA-2 were related to *Streptomyces mutabilis* NBRC 12800^T (100% of similarity) and *Streptomyces cyaneofuscatus* JCM 4364^T (100% of similarity), respectively.

1. Introduction

Rhizoctonia solani is one of the most important soil-borne fungal pathogens, which develops in both cultured and non-cultured soils (Coa et al. 2004). It lives in the soil in the form of sclerotia and does not generate asexual spores (Huang et al. 2011). Damping-off of seedlings is the most common disease caused by *R. solani* (Moussa 2002). It has a wide host range and causes disease in a variety of crops, such as lawn grass (Parmeter et al. 1969), tomato (Coa et al. 2004), cucumber (Coa et al. 2004) and sugar beet (Sadeghi et al. 2006).

Chemical fungicides are often used when losses from *R. solani* are substantial. They have fungicidal activity against the pathogen or they are converted into toxic derivatives by the pathogen or host plant tissue (Grissbuhler et al. 1982). The intensive use of chemical fungicides has not only created problems of fungicide resistance and increased contamination of the soil, but may also have adverse high toxicity on microbial communities and a degradative effect on the ozone layer. In addition, chemical controls are not completely effective, and *Rhizoctonia* disease remains a persistent problem (Gees and Coffe 1989; Huang et al. 2011).

The success of *Bacillus subtilis* as a potential biocontrol agent encouraged research into new microbial agents as alternatives to chemical control compounds. In recent years, the biocontrol of plant diseases, particularly using antibiotic metabolites of filamentous bacteria, has been put forward as an alternative to chemical control agents (Dhanasekaran et al. 2005; Huang et al. 2011). The role of actinomycetes in the biocontrol of soil-borne plant pathogens has been demonstrated against various pathogens such as *Fusarium* spp. (Sabaou and Bounaga 1987; Gopalakrishnan et al. 2011), *Phytophthora* spp. (Shahidi Bonjar et al. 2006), *Pythium* spp. (Hamdali et al. 2008), *Rhizoctonia* spp. (Sadeghi et al. 2006), and *Verticillium* spp. (Meschke and Schrempf 2010).

Actinomycetes can occur in the plant rhizosphere soil and exercise an antagonistic and competitive effect on the microbial communities. They have the ability to produce active compounds, such as antifungal and antibacterial antibiotics or plant growth

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regulators (PGRs), that have been developed for agricultural uses (Suzuki et al. 2000; Ilic et al. 2007). They have also been used as commercially formulated biocontrol agents of plant diseases such as *Streptomyces griseoviridis* cells used to protect crops against infections by *Fusarium* spp. and *Alternaria* spp. (Lahdenperä et al. 1991). In addition to their ability to inhibit plant pathogens, some actinomycetes are also known to form close associations with plants, colonize their internal tissues without causing disease symptoms, and promote their growth (Kunoh 2002).

Several native plants have successfully adapted to the stressful conditions of the Algerian Sahara, especially the poor sandy soil and the drought of the arid climate. They grow and colonize these areas annually without human intervention (Pouget 1980). The success of natural regeneration from seeds and the vigorous growth of native plants under the harsh conditions of the Sahara could suggest a contribution of endophytic microbes to the biological protection of germinated seeds against soil-borne pathogens and to the promotion of plant growth. In recent work, we initiated a study to highlight the role of endophytic microbes from these plant species as potential biocontrol agents against damping-off disease caused by soil-borne pathogens and as plant growth promoters (Goudjal et al. 2013).

The aims of the present study were to isolate endophytic actinomycetes from healthy Saharan plants and bring out their potential role on the biocontrol of *R. solani* damping-off and on plant growth promoting activities for tomato seedlings. In order to select the most interesting strains for the biocontrol of *R. solani* damping-off, a commercial chemical agent was used as a control.

2. Materials and methods

2.1. Sample collection and endophytic actinomycetes isolation

Healthy native plants (Aristida pungens, Cleome arabica, Solanum nigrum, Panicum turgidum, *Astragallus armatus*, *Peganum harmala*, *Hammada scoparia* and *Euphorbia helioscopia*) were collected from the Laghouat region in the northern Algerian Sahara (33°44′ N, 2°47′ E) in March 2011. The selection of plant species for endophyte isolation was based on their abundance and adaptation to the poor sandy soil and arid climatic conditions of the Algerian Sahara. Five healthy root samples were harvested from each plant species. No record indicates that these plants have ever been studied for endophytic actinomycetes isolation previously.

Endophytic actinomycetes were isolated according to the method of Taechowisian et al. (2003). The culture medium used was chitin-vitamin agar (Hayakawa and Nonomura 1987) supplemented with cycloheximide (80 mg l^{-1}) and nalidixic acid (15 mg l^{-1}) to suppress the growth of fungi and Gram-negative bacteria, respectively.

2.2. Effectiveness of the surface-sterilization protocol

To prove that epiphytic actinomycetes could not grow after the surface-sterilization of the roots and that all the isolated actinomycetes were endophytic, the method described by Schulz et al. (1993) was used.

2.3. Identification of the endophytic actinomycetes

Endophytic actinomycetes belonging to the *Streptomyces* genus were identified based on cultural and micromorphological characteristics according to the methods of Shirling and Gottlieb (1966) and Goodfellow and Simpson (1987).

In order to distinguish the *Streptomyces* genus, the cell wall type was determined by a chemical study based on the occurrence of diaminopimelic acid isomers (Becker et al. 1964).

Identification of the highest potential isolates (CA-2 and AA-2) on biocontrol of *R. solani* damping-off and plant growth promoting activity for tomato *cv.* Marmande seedlings was confirmed by the 16S rRNA gene sequence analysis. Genomic DNA was prepared according to the CTAB method (Liu et al. 2000). The 16S rRNA gene sequence was amplified by the PCR method as used by Boubetra et al. (2013). The PCR products obtained were sent to the MilleGen Company (Toulouse, France) for sequence determination. The16S rRNA sequences have been deposited in the GenBank data library and assigned the accession numbers KC414006 and KC414004 for CA2 and AA2 strains, respectively. The obtained sequences were compared with sequences present in the public sequence databases and with EzTaxon tools (Chun et al. 2000).

2.4. In vitro antagonism assay

The streak method described by Boubetra et al. (2013) was used to test the antifungal activity of the endophytic actinomycete isolates against an indigenous soil-borne pathogenic strain of *R. solani* LRS1 isolated from tomato fields in the Laghouat region (33°46′ N, 2°50′ E). Inhibition zones were evaluated as follows: (<5 mm) no inhibition, (5-9 mm) weak inhibition, (10-19 mm) moderate inhibition, and (≥20 mm) strong inhibition.

2.5. In vivo biocontrol trials

2.5.1. Soil properties

The sandy soil used to test the effectiveness of the antagonistic isolates to control damping-off of tomato *cv.* Marmande was collected from a tomato field in the Laghouat region (33°46′ N, 2°50′ E). Its physicochemical and biological characteristics were: pH 7.8, total organic matter (1.8%), C/N (9.3), phosphate (0.07‰), potash (0.3‰), $CaCO_3$ (1.1%) total aerobic bacterial count 1.52×10^8 CFU g^{-1} and total fungal count 3.7×10^4 CFU g^{-1}. Soil was sterilized three times in an autoclave at 120 °C for 60 min on three consecutive days.

2.5.2. Preparation of microbial suspensions

Actinomycete suspensions were prepared as described by Errakhi et al. (2007). Tween-80 solution (0.05%) was used to recover the spores, which were adjusted to 10^6 CFU ml^{-1} by the same solution using the Thomas cell. A pathogen suspension was prepared by growing *R. solani* in PDA dishes at 25 °C for 10 days. Spores were directly recovered in sterile distilled water and adjusted to 10^5 CFU ml^{-1} in the same way.

2.5.3. Coating of tomato seeds

Tomato seeds were surface-sterilized using the method of Coa et al. (2004). Sterilized seeds were coated separately by soaking for 30 min in the actinomycete spore suspensions and then dried under a laminar flow hood. Actinomycete spores on the coated seeds yielded approximately 10^7 CFU g^{-1}.

2.5.4. Preparation of infested soils

Sterilized and non-sterilized soils were infested according to a method similar to that used by Dhanasekaran et al. (2005). Soils were conditioned in plastic pots (12 cm high × 10 cm in diameter) and infested with 10 ml of pathogen suspension diluted in 90 ml sterile distilled water (100 ml sterile distilled water for uninfected soils). The pots were covered with plastic film and incubated for one week at room temperature to promote pathogen growth. *R. Solani* density in the infested soils was evaluated at 10^4 CFU g^{-1}.