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Biocontrol of *Rhizoctonia solani* damping-off disease in cucumber with *Bacillus pumilus* SQR-N43

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ABSTRACT

Biological control is an efficient and environmentally friendly way to prevent damping-off disease. Micrographs were used to investigate the ability of *Bacillus pumilus* (*B. pumilus*) SQR-N43 to control *Rhizoctonia solani* (*R. solani*) Q1 in cucumbers. The root colonization ability of *B. pumilus* SQR-N43 was analyzed *in vivo* with a green fluorescent protein (GFP) tag. A pot experiment was performed to assess the *in vivo* disease-control efficiency of *B. pumilus* SQR-N43 and its bio-organic fertilizer. Results indicate that *B. pumilus* SQR-N43 induced hyphal deformation, enlargement of cytoplasmic vacuoles and cytoplasmic leakage in *R. solani* Q1 mycelia. A biofilm on the root surface was formed when the roots were inoculated with 10^7–10^8 cells g^{-1} of soil of GFP-tagged *B. pumilus* SQR-N43. In the pot experiment, the biocontrol reduced the concentration of *R. solani*. In contrast to applications of only *B. pumilus* SQR-N43 (N treatment), which produced control efficiencies of 23%, control efficiencies of 68% were obtained with applications of a fermented organic fertilizer inoculated with *B. pumilus* SQR-N43 (BIO treatment). After twenty days of incubation, significant differences in the number of CFUs and the percentage of spores of *B. pumilus* SQR-N43 were recorded between the N treatment (2.20×10^7 CFU g^{-1} of soil and 79%, respectively) and the BIO treatment (1.67×10^8 CFU g^{-1} of soil and 52%, respectively). The results indicate that *B. pumilus* SQR-N43 is a potent antagonist against *R. solani* Q1. The BIO treatment was more effective than the N treatment because it stabilized the population and increased the active form of the antagonist.

1. Introduction

Rhizoctonia solani (*R. solani*) is an important fungal pathogen (Baker 1970) that lives in the soil in the form of sclerotia and does not generate asexual spores. It has a wide host range and distribution and causes sheath blight in some field crops, such as corn (Ogoshi 1987), rice (Ou 1985), lawn grass (Parmeter et al. 1969) and cucumber (Strashnov et al. 1985). Currently, *Rhizoctonia* disease is managed by cultural practices, such as crop rotation with grains, and methods that minimize prolonged contact of the plant with the pathogen, such as planting in warmer, drier conditions to promote rapid sprout emergence and promptly removing tubers from the field (Secor and Gudmestad 1999). Chemical fungicides are often used when losses from *R. solani* are substantial (Parry 1990). However, current cultural and chemical controls are not completely effective, and *Rhizoctonia* disease remains a persistent problem.

Biological control is an efficient and environmentally friendly way to prevent damping-off disease. Many microbial species such as *Trichoderma atroviride* (Reithner et al. 2007), *Trichoderma harzianum* (Hadar et al. 1979), *Pseudomonas fluorescens* (Nagarajkumar et al. 2004) and *Bacillus subtilis* (Asaka and Shoda 1996) have been shown to effectively control *R. solani*. However, few reports have been published that document the ability of *Bacillus pumilus* (*B. pumilus*) to control damping-off disease.

The successful survival and colonization of beneficial antagonists are essential for the biocontrol of diseases caused by soil-borne pathogens. Previous reports have demonstrated that the control efficiencies of *Pseudomonas* spp. and *Bacillus* spp. were directly related to the ability of these microbes to colonize roots (Cavaglieri et al. 2005; Ji et al. 2008). However, the population dynamics and colonization pattern of *B. pumilus* in situ has not been studied in detail (Akhtar et al. 2010).

Reporter gene technology is indispensable for monitoring bacteria in environmental samples (Ramos et al. 2000). Various markers, such as lacZ and luxAB, have been used to identify cells and assess physiological activities in the rhizosphere (Unge et al. 2001). Green fluorescent protein (GFP), isolated from the jellyfish *Aequorea victoria*, is the most revolutionary reporter in biology (Chalfie et al. 1994). Unlike other reporter genes such as β-glucuronidase, GFP requires only UV or blue light and oxygen and does not require cofactors or destructive sampling of substrates to localize activity (Chelius and Triplett 2000; Rajasekaran et al. 2008). Because of these advantages, GFP has been widely used to investigate bacterial growth, localization, distribution and colonization in complex

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samples such as soil and plant tissues (Liu et al. 2006; Njoloma et al. 2006; Neveu et al. 2007; Cao et al. 2011).

Several studies have indicated that organic substrates could provide functional microbes with nutrients that increase their competitiveness in the soil (Wu et al. 2009; Ling et al. 2010; Chen et al. 2011). We developed a novel bio-organic fertilizer (BIO) by fermenting mature compost with *B. pumilus* N43 (N43). The objective of this study was to evaluate the effect of a combination of organic fertilizer and an antagonistic microorganism to control *R. solani* in cucumbers. Quantification real-time PCR and denaturing gradient gel electrophoresis (DGGE) were used to investigate variations in the populations of the pathogen and the soil microbial community during the biocontrol process. To investigate its root colonization ability, cells of *B. pumilus* SQR-N43 (N43) were labeled with GFP, and localization of these bacteria on cucumber roots was monitored with confocal laser scanning microscopy (CLSM). An assay to assess bacterial–fungal interactions was also performed *in vitro*.

2. Materials and methods

2.1. Strains

B. pumilus SQR-N43 strain was isolated from the cucumber rhizosphere, identified in preliminary experiments and stored on a nutrient agar (NA) medium at 4 °C. The *R. solani* Q1 was obtained from the Institute of Vegetables and Flowers at the Chinese Academy of Agricultural Sciences in Beijing, China.

2.2. Dual culture

A bacterial isolate of N43 was grown in nutrient broth on a rotary shaker at 30 °C and 170 rpm for 24 h. The suspension was centrifuged in sterile 50-ml plastic tubes at 6000 rpm for 10 min. The pellets were re-suspended in sterile distilled water to obtain a final concentration of 10^8 cells ml^{-1} (OD = 0.5) at 600 nm, which was measured with the viable plate count and optical density methods. One 5-mm disk of pure *R. solani* Q1 culture was placed in the center of a Petri dish containing potato dextrose agar (PDA). Four drops of the bacterial suspension were placed around the fungal inoculums at a distance of 2 cm. In the control, sterile distilled water was used in place of the suspension. Plates were incubated for 48 h at 28 °C. Each experiment was conducted in triplicate and repeated at least three times.

In dual cultures, *R. solani* Q1 mycelium on the edge of the inhibitory halo was used to observe the effects of N43. An optical microscope (OLYMPUS CX21, Tokyo, Japan) and a scanning electron microscope (HITACHI S-3000 N, Tokyo, Japan) were used for the observations.

2.3. Construction and root colonization ability of GFP-tagged N43

2.3.1. Construction of GFP-tagged N43

The plasmid pHAPII (GenBank accession number HM151400), an *Escherichia coli–Bacillus subtilis* shuttle vector that contained the *hapII*-controlled GFP gene, was constructed in our laboratory. Electroporation was used to construct the mutant (Cao et al. 2011).

2.3.2. Seedling incubation and inoculation

Cucumber (*Cucumis sativus* L.) seeds of the cultivar JinChun 5 were surface-sterilized in 2% NaClO for 3 min and 75% ethanol for 2 min and then rinsed 3 times in sterile water. The seeds were then germinated on 9-cm plates covered with sterile wet filter paper at 25 °C for 48 h. GFP-tagged N43 cells were grown in Luria Bertani (LB) medium containing 20 μg ml^{-1} of kanamycin for 24 h. The bacteria were harvested by centrifuging at 5000 × *g* for 10 min and washing twice with phosphate-buffered saline (PBS) with a pH 7.0. The soil used in the experiment was inoculated with the bacterial suspension to obtain final concentrations of 10^5, 10^6, 10^7 and 10^8 cells per g of soil. After inoculation, germinated seeds were transferred to the soil, irrigated with 1/2 Hoagland medium and incubated in a growth chamber at 25 °C under a 16-h light regimen.

2.3.3. Microscopy

The pattern of bacterial colonization on the roots was monitored two weeks after transplantation. Root samples were cut to lengths of 0.2–0.5 cm and visualized using a CLSM (Leica Model TCS SP2, Heidelberg, Germany) with excitation wavelengths of 488 nm. Emitted light in the range of 500–600 nm was collected for the GFP. Images were generated with version 2.61 of the Leica confocal software. After being soaked for 15 min in a suspension containing 10^8 cells of GFP-tagged N43 per ml, the roots were immediately observed to establish a positive control.

2.4. In vivo antagonism of GFP-tagged N43 against R. solani

To determine the ability of N43 to protect roots against *R. solani* Q1 *in vivo*, cucumber seeds were treated with the GFP-tagged bacteria prior to exposure to the phytopathogenic fungi. The seeds were prepared as described above and then pre-germinated in the dark on sterile wet filter paper in Petri dishes for 4 days. The pre-germinated seeds were immersed in a suspension of GFP-tagged N43 with a concentration of 10^8 cells ml^{-1} and then germinated in water agar plates for 2 days. The plates were then inoculated with *R. solani* Q1 and incubated at 25 °C in the dark. Four days after inoculation with the pathogen, the rhizosphere of the cucumber seedlings was observed with a fluorescence microscope (OLYMPUS MVX10, Tokyo, Japan). Five replicates were performed for each treatment. In the control treatment, seeds were germinated on water agar plates with *R. solani* alone.

2.5. Pot experiment

2.5.1. Cucumber seedling, soil and fertilizer for pot experiments

Cucumber seeds were prepared as described above. The soil used in the pot experiment was solarized to minimize the influence of other microorganisms and air-dried prior to the experiment. The dried soil had the following properties: pH 6.7, 0.9% organic matter, 0.1% nitrogen and 4.4% H_2O. The organic fertilizer (OF) used in the pot experiment was an amino acid fertilizer made from oil rapeseed cakes that had been enzymatically hydrolyzed by aerobic microbial fermentation at <50 °C for seven days (Zhang et al. 2008). The fertilizer contained 44.2% organic matter, 12.9% amino acids, small molecular peptides and oligo peptides. The nutrient content was 4.4% N, 2.3% P_2O_5 and 0.7% K_2O.

2.5.2. Pathogen, N43 and N43-fermented organic fertilizer (BIO) preparation

Erlenmeyer flasks (250 ml) containing 100 ml of potato dextrose broth (PDB) were incubated with 1 cm^2 discs of the actively growing mycelia of *R. solani* Q1. The inoculated flasks were incubated at 28 °C for 4 days without shaking. The mycelia were then collected by filtration through gauze and washed with distilled water.

The BIO product used in the experiments was obtained by aerobically fermenting a mixture of the amino acid fertilizer with N43 for 5 days at <45 °C. The cell suspension of N43 was prepared using NB medium and homogenized in distilled water. The mixture was thoroughly mixed and maintained at 40–45% moisture during the fermentation stage. At the end of the fermentation stage, the density of N43 was 10^9 CFU per gram of BIO (dry weight). The BIO product was stored at 4 °C prior to use in experiments.