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Involvement of lipopeptide antibiotics and chitinase genes and induction of host defense in suppression of Fusarium wilt by endophytic *Bacillus* spp. in tomato

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ABSTRACT

Five endophytic *Bacillus* spp. isolated from surface-sterilized stem tissues of wild Solanaceous species, i.e. *Datura metel*, *Solanum nigrum* and *S. elaeagnifolium*, were screened for their *in vitro* and *in vivo* antagonistic potential against *Fusarium oxysporum* f. sp. *lycopersici* (FOL). Pathogen sporulation was totally suppressed by these five strains and its mycelial growth was significantly decreased using bacterial whole-cell suspensions and cell-free culture filtrates. Extracellular metabolites remained effective after heating at 50–100 °C with a decline in activity with treatments involving temperatures at 100 °C, proteinase K addition, and pH adjustments to 2 or 12. Chitinase and lipopeptide antibiotic genes were detected using PCR amplification. The five strains produced salicylic acid when grown in succinate medium, with the greatest production exhibited by *B. subtilis* str. SV41. In planta application of *Bacillus* spp. cell-free culture filtrates and whole-cell suspensions significantly decreased Fusarium wilt severity by 87–100% and enhanced tomato growth by 38–80%, compared to FOL-inoculated and untreated controls. The two *Bacillus* strains exhibiting the strongest decrease in Fusarium wilt severity (*B. subtilis* str. SV41 and *B. amyloliquefaciens* subsp. *plantarum* str. SV65) were further evaluated for their ability to induce systemic resistance (ISR) in FOL-infected and uninfected tomato plants. Using quantitative RT-PCR, the expression of the acidic PR-1 and PR-3 genes as well as the lipoxygenase (LOXD) gene were significantly induced in plants treated with *B. amyloliquefaciens* subsp. *plantarum* str. SV65. This effect was observed in both FOL-inoculated and uninoculated plants.

1. Introduction

Fusarium oxysporum f. sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hans (FOL) is a highly destructive soil-borne pathogen causing wilt disease in both greenhouse and field-grown tomatoes and leads to greatly reduced yields (Gajanana et al., 2006). FOL infection leads to severe damage in stems and leaves resulting in leaf yellowing, vascular browning, plant wilting, stunting, and eventual death of the whole plant (Lim et al., 2006). Crop rotation, chemical control and solarization, techniques extensively used worldwide, have failed to effectively suppress Fusarium wilt. Indeed, due to the development of fungicide resistance and to the emergence of new physiological races of FOL, many fungicides and resistant cultivars are gradually becoming ineffective against this disease (Ge et al., 2004).

Chemical control of tomato Fusarium wilt is hampered by the spread of the pathogen through plant vascular tissues. Therefore, an attractive and environmentally safer alternative is the potential use of endophytic microorganisms that limit disease incidence and severity by preventing the fungus from spreading through the plant

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vascular system. These endophytes are defined as bacteria or fungi that reside in plants tissues without causing negative effects on plant growth and can be isolated from the plant after surface disinfection (Hallmann et al., 1997; Gaiero et al., 2013).

Bacillus species are reported to be endophytes of higher plants (Bacon and Hinton, 2002; Li et al., 2012; Kalai-Grami et al., 2014) and frequently colonize wild Solanaceous species (Bhuvaneswari et al., 2013; Achari and Ramesh, 2014; Santhanam et al., 2014). Recently, we have reported the isolation and identification of endophytic *Bacillus* strains from three wild Solanaceous species, *N. glauca*, *D. stramonium* and *S. elaeagnifolium* (Aydi Ben Abdallah et al., 2016a, 2016c, 2016d). Using whole cell cultures of endophytic *B. cereus* str. S42, *B. mojavensis* str. S40 and *B. tequilensis* str. SV104, we showed that these strains have a phytoprotective potential, acting both as biocontrol agents against FOL, as well as the capability to act as biofertilizers, by promoting the growth of tomato plants. In the present study, the suppressive effect of extracellular metabolites from *B. tequilensis* str. SV104 obtained from *S. elaeagnifolium*, *B. tequilensis* str. SV39, *B. subtilis* str. SV41 and *B. methylotrophicus* str. SV44 originally isolated from *D. metel* and *B. amylolequifaciens* subsp. *plantarum* str. SV65 recovered from *S. nigrum* towards tomato Fusarium wilt was newly tested. In addition, the efficacy of the *in vitro* antifungal activity of cell-free culture filtrates from the five *Bacillus* spp. strains was assessed following heat, enzymatic degradation and pH modification treatments.

These bacterial strains inhibit pathogen growth through the production of antibiotics, cell wall-degrading enzymes, competition for nutrients and minerals and/or inducing systemic resistance (Lugtenberg et al., 2013). In fact, chitinolytic and proteolytic activity were detected in *B. mojavensis* str. S40 (Aydi Ben Abdallah et al., 2016a), *B. cereus* str. S42 (Aydi Ben Abdallah et al., 2016c) and *B. tequilensis* str. SV104 (Aydi Ben Abdallah et al., 2016d), which inhibited FOL growth by 39–64% and induced an inhibition zone against FOL of about 12–20 mm when used as whole cell cultures. The secreted chitinolytic enzymes, which hydrolyze the β-1,4-glycosidic bonds in chitin, the main component of the cell wall of fungi, are important contributors to the antagonistic activity of bacterial strains against fungal plant pathogens (Kalai-Grami et al., 2014). In this study, chitinase-producing strain, *B. tequilensis* str. SV104, in chitin-agar medium (Aydi Ben Abdallah et al., 2016d) and other *Bacillus* species i.e. *B. tequilensis* str. SV39, *B. subtilis* str. SV41, *B. methylotrophicus* str. SV44 and *B. amylolequifaciens* subsp. *plantarum* str. SV65, which have been found to produce chitinase in agar plate (unpublished data), were assessed for the expression of chitinase (*ChiA*) gene. In addition, several endophytic *Bacillus* species have been considered to be natural factories of biologically active compounds such as lipopeptide antibiotics (LPs) including surfactin, iturin, and fengycin families (Ongena and Jacques, 2008; Malfanova et al., 2012). In view of these previous findings, our five endophytic *Bacillus* spp. strains were assessed for the expression of LPs genes encoding for surfactin, fengycin, bacillomycin, and iturin. More recently, certain LPs were identified as elicitors that are able to indirectly protect plants through the stimulation of inducible defense mechanisms (Han et al., 2015).

Plants have several defense mechanisms against pathogen attacks. Induced resistance to pathogens can be subdivided into two broad categories. Systemic acquired resistance (SAR), which is mediated via a salicylic acid (SA)-dependent process and associated with the production of Pathogenesis Related (PR) proteins (Durrant and Dong, 2004). The second type is induced systemic resistance, which is conferred by non-pathogenic root colonizers (van Loon et al., 2006) and is mostly dependent on the jasmonate (JA) and/or ethylene (ET) signaling pathways rather than SA (Pieterse et al., 2009). In this study, the production of salicylic acid by five *Bacillus* strains was assessed. Furthermore, other mechanisms displayed in induction of systemic resistance in plants such as the expression of lipooxygensase (LOXD) gene and PRs (PR1, PR2 and PR3) genes were assessed in this study.

2. Materials and methods

2.1. Plant material

Tomato cultivar Rio Grande, susceptible to FOL races 2 and 3 (Barker et al., 2005) was used throughout this study. Seeds were sown in 77-plug trays filled with previously sterilized peat® (Floragard Vertriebs GmbH für gartenbau, Oldenburg). Trays were watered regularly to avoid drought stress and kept under greenhouse conditions (20–30 °C with a 16 h light/8 h dark cycle and 60–70% relative humidity). Seedlings at the two-true leaf growth stage were used in all the bioassays.

2.2. Pathogen culture

The strain of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) used in this study was originally isolated from tomato plants showing typical symptoms of Fusarium wilt (Aydi Ben Abdallah et al., 2016b). This strain was re-isolated from artificially inoculated tomato cv. Rio Grande plants fulfilling Koch's postulates (Aydi Ben Abdallah et al., 2016c). It was cultured on Potato Dextrose Agar (PDA) and incubated at 25 °C for 7 days before use.

2.3. Bacillus strain culture

Five endophytic strains of *Bacillus* spp. (*B. tequilensis* str. SV39, *B. subtilis* str. SV41, *B. methylotrophicus* str. SV44, *B. amylolequifaciens* subsp. *plantarum* str. SV65, and *B. tequilensis* str. SV104), recovered from internal stem tissues of wild Solanaceous species, were selected for use in this study because they were the most effective in reducing Fusarium wilt severity on tomato plants inoculated with FOL using whole cell suspensions and cell-free culture filtrates (data not shown). The accession numbers and isolation sources of the five strains tested are listed in Table 1. Isolation procedure, characterization and identification using 16S rDNA gene sequencing were described in previous works (Aydi Ben Abdallah et al., 2015; Aydi Ben Abdallah et al., 2016d). Before being used in bioassays, stock cultures maintained at −20 °C in Nutrient Broth (NB) supplemented with 40% glycerol were grown on Nutrient Agar (NA) medium and incubated at 25 °C for 48 h.

2.4. Endophytic colonization ability of Bacillus spp. on tomato plants

Five endophytic strains of *Bacillus* spp. were transferred to NA amended with 100 μg/ml (w/v) streptomycin sulphate and 100 μg/ml (w/v) rifampicin (Chen et al., 1995). The original bacterial strains exhibiting resistance to these two antibiotics were chosen in order to follow their presence in tomato stems after re-isolation in NA medium containing both antibiotics. The wild type strain was inoculated in tomato cv. Rio Grande plants at two-true-leaf stage by dipping roots for 30 min in water suspensions of bacterial cells adjusted to 10^8 cells/ml. The bacterial suspensions were prepared by scraping bacterial colonies, previously grown in NA for 48 h, in sterile distilled water (SDW). Control plants were dipped in SDW only.

Seedlings were transplanted into individual pots (12.5 cm × 14.5 cm) containing commercialized peat. Five replicates of one seedling each were used for each individual treatment and the whole experiment was conducted twice. After 60 days of