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Evaluation of *Bacillus* strains isolated from solanaceous phylloplane for biocontrol of *Alternaria* early blight of tomato

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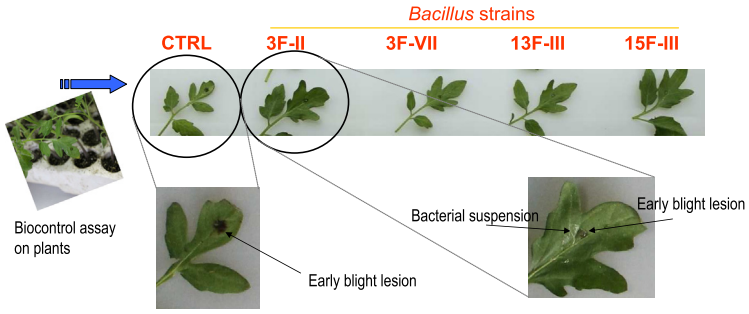
HIGHLIGHTS

- Ninety-three phylloplane bacteria were screened for antagonism against *Alternaria alternata*.
- Four *Bacillus* strains capable of control early blight of tomato were selected.
- Antibiosis and fungi-stasis can be involved in antagonistic mechanisms.
- Hyphal structures damaged by bacterial secretion were observed in light microscope.

GRAPHICAL ABSTRACT



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ABSTRACT

Bacillus biological control agents are powerful alternatives to chemical fungicides for suppressing plant diseases caused by fungi. In this work, 93 strains of spore-forming bacteria isolated from solanaceous phylloplane, were screened for *in vitro* antibiotic activity against *Alternaria alternata*, causal agent of tomato early blight. The twenty most active strains were characterized for their morphological and physiological traits (Gram reaction, production of endospores, antifungal volatile compounds and siderophores) and by M13-PCR DNA fingerprinting. *Planta* bioassays performed with four selected strains, were capable to decrease severity of *Alternaria* disease on tomato. These antagonistic bacteria were identified by 16S-rRNA partial gene sequencing, and results assigned them to strains related to *Bacillus* species. The evident inhibition zone observed in dual culture plates, suggested an antibiosis-like mechanism. While, API-ZYM enzymatic profiles indicated that strains could be potential ecological competitors. Consistently, light microscopy revealed the occurrence of *Bacillus*-induced malformations in the fungus's vegetative structures, probably caused by secreted compounds.

1. Introduction

Early blight caused by *Alternaria alternata* (Fr.) Keissler, is an economically important disease of tomato. It is a production-limiting factor, since the fungus mainly affects the leaves, causing a significant loss of net photosynthetic surface area. In severe cases, the disease can cause complete defoliation of the plants, with a negative impact on the yield. Epidemics can occur in climate regions with heavy rainfall, high humidity and fairly high temperatures in the range from 24 to 29 °C (Çalış and Topkaya, 2011). Effective control of early blight is essential, and is consequently entrusted primarily to synthetic chemicals. Policies to reduce the risks relating to the use of fungicides in agriculture are driving efforts to find suitable and sustainable alternatives to these traditional pest control measures.

Recently, for example, two microbial antagonists, the bacteria *Paenibacillus lentimorbus* (Khan et al., 2012) and the yeast

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Rhodotorula glutinis (Yan et al., 2014) has been experimented for the non-chemical reduction of *Alternaria* disease on tomato leaves and cherry fruits, respectively. Actually, biological control is one of the most viable environment and health-friendly approaches for replacing chemical fungicides in suppressing plant diseases caused by fungal pathogens (Pal and McSpadden Gardener, 2006).

Living bacterial microorganisms exhibiting antagonistic properties are being studied and selected to formulate innovative biopesticides. Among them, the large family of the genus *Bacillus* encompasses various species that include several of the biocontrol strains described to date (Jacobsen et al., 2004). Thanks to their numerous valuable traits, members of this Gram-positive bacterial group represent one of the major sources of microbial biological control agents (BCAs). These beneficial microorganisms are recognized as being non-pathogenic for humans, forming heat- and desiccation-resistant spores and secreting antibiotics, enzymes and other antagonist molecules, and possibly acting as mycoparasites and competing for ecological niches and nutrients (Cawoy et al., 2011).

This bacterial group is already well represented commercially. Most (70%) of the *Bacillus* strains exploited as biopesticides are *Bacillus thuringiensis*, used specifically for insect pest control. Other members of the family most frequently used in applications for combating disease are *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Bacillus pumilus* (Ongena and Jacques, 2007). Microbiological control tools still contribute for less than 10% of all pesticide sales; however, the search for novel antagonists is still extremely important with a view to offering additional biological disease control options.

Their adaptability to extreme conditions makes *Bacillus* members particularly widespread in various environments, so they are versatile in biocontrol applications. This property can be further exploited to select BCAs from the same ecological niche in which pathogenesis takes place, to make pest control measures more effective (Köhl, 2009). In previous studies performed by Kumar et al. (2012) and Li et al. (2012), the authors reported biocontrol activity of rhizosphere-competent *Bacillus* strains against soil-borne phytopathogens.

The aim of the present work was to isolate and characterize new heat-resistant spore-forming bacterial strains from solanaceous phylloplane, select those proving *in vitro* effective against *A. alternata* and test the efficacy of these candidate antagonists in the control of early blight leaf infections.

2. Materials and methods

2.1. Isolation of bacteria from the solanaceous phylloplane

To isolate spore-forming bacteria, pieces of leaf (5 g) collected from healthy solanaceous plants, such as tomato, eggplant and pepper cultivated in different cropping systems at CRA experimental farm of Battipaglia (Southern Italy), were washed for 1 h by stirring them on a rotary shaker in 100 ml of physiological saline solution (0.85% NaCl) (Hwanhlem et al., 2014). Then, 50 ml of the suspension were centrifuged at 10,000*g* for 10 min and the precipitate was suspended in 1 ml of water. Aliquots of the final suspension were heated to 90 °C for 10 min, and then inoculated on nutrient agar (Sadfi et al., 2001) at 28 °C. Some representative colonies were selected from the countable plates and streaked twice on new NA plates to obtain pure colonies. Purified bacterial strains were stored at −80 °C in 20% glycerol nutrient broth.

2.2. Physiological, morphological and molecular characterization of the bacteria

All bacteria isolated (93) were characterized for *in vitro* antibiosis activity using a qualitative assay against *A. alternata* strain I 205 (CRA collection). This test was done on PDA plates using a dual culture technique (Cazorla et al., 2007). Each plate was inoculated with a plug (Ø 5 mm) of active growing mycelia in the middle, and bacterial strains were inoculated around the edge of the plates. After a week of incubation at 25 °C, bacteria showing a distinctive inhibition area were collected and stored. Then, only the best twenty potentially antagonistic bacterial strains were first characterized for: Gram reaction by Ryu test (Ryu, 1940); cell morphology and spore formation by visualization under a light microscope; production of antifungal volatile compounds against *A. alternata* using the septed-plate technique (Zheng et al., 2013); production of siderophore-like substances on nutritive substrate without iron (Scher and Baker, 1982), after adding 100 μg ml^{-1} of cycloheximide (Fluka, Buchs, Switzerland) (Zaccardelli et al., 2013). Then, PCR fingerprinting was done by M13-PCR, as described by Pane et al. (2012a), using M13-primer (5′ GAGGGTGGCGGTTCT 3′). The banding patterns obtained after electrophoresis were visualized under UV light and computed by cluster analysis on binary matrices.

2.3. Quantitative assessment of antagonistic activity of the bacterial strains

Bacteria showing antibiosis activity were assayed in a quantitative plate challenge, performed according to Boulter et al. (2002). A 5-mm plug was transferred from the edge of an actively growing fungal colony to the center of a PDA Petri dish (∅ 90 mm), in which pure cultures of bacterial strains were streaked around the edge. While, plates that were not streaked with bacteria but inoculated with the fungus, were used as reference control. Plates were inoculated in triplicate and incubated at 25 °C. The diameter of the mycelia was measured daily until the fungi reached the edge of the control plates. The percentage of fungal growth inhibition due to the antagonistic activity of the bacteria, were calculated using the following formula: $100 \times$ [(diameter in control plate − diameter in treated plate)/diameter in control plate].

2.4. Biocontrol of Alternaria blight on tomato

One-month-old tomato plants (*Solanum lycopersicum* L. cv. Corbarino) were used to test the bacteria's ability to control, *in vivo*, *Alternaria* early blight disease. Two-week-old *A. alternata* PDA culture was used to produce the conidial inoculum by collecting conidia in a water suspension, with a concentration adjusted to 1×10^5 conidia ml^{-1}. Plants were infected by inoculating a drop (5 μl) of conidial suspension on one last developed true leaf *per* plant, previously micro-wounded with a sterile needle (Pane et al., 2012b). Two separate biocontrol assays were carried-out in order to study the effect of the rate and the timing of antagonists application on disease control. The first assay was carried-out by co-inoculating a drop (5 μl) of a bacterial water suspension adjusted to ten-fold increasing concentrations 1×10^6–1×10^9 cells ml^{-1} with the pathogen conidia. In the second one, biocontrol treatments were performed by pipetting a drop (5 μl) of water-suspended bacteria (1×10^9 cfu ml^{-1}) 1-day-before, at the same time and 1-day-after the pathogen inoculation, performed in the correspondence of treating points. Thus, all plants were simultaneously infected as described above and were incubated all together under the same conditions.

In both assays, plants only infected with the pathogen were used as reference control. Plants were kept into a plastic chamber to maintain >98% relative humidity and incubated in a climatic room at 25 °C, in a completely randomized design. After a week, the diameter of the lesions was measured and expressed as percentage of those recorded on the control plants. The experimental design included four BCA treatments, applied at rate and timing