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Characterization of new bacterial biocontrol agents *Acinetobacter*, *Bacillus*, *Pantoea* and *Pseudomonas* spp. mediating grapevine resistance against *Botrytis cinerea*

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Abstract

A collection of 282 bacterial isolates from the rhizosphere and different organs of healthy field-grown grapevine plants was obtained and screened for their ability to protect grapevine leaves against *Botrytis cinerea*, the causal agent of gray mold. Twenty-six strains effectively controlled *B. cinerea* infections on leaves. After phenotypic and molecular analysis, seven strains were identified as *Pseudomonas fluorescens* PTA-268 and PTA-CT2, *Bacillus subtilis* PTA-271, *Pantoea agglomerans* PTA-AF1 and PTA-AF2, and *Acinetobacter lwoffii* PTA-113 and PTA-152. In vitro antifungal experiments showed that from these seven strains, only PTA-AF1 and PTA-CT2 exhibited a direct antagonism against *B. cinerea*. Furthermore, the biocontrol activity of the seven bacteria was associated with differential induction of defense-related responses lipoxygenase, phenylalanine ammonia-lyase and chitinase in grapevine leaves. Our results show that the selected bacteria can efficiently protect grapevine leaves against gray mold disease through an induction of plant resistance and in some cases by an additional antagonistic activity.

Keywords: Biocontrol; Grapevine; Gray mold; Induced resistance; Rhizobacteria

1. Introduction

Grapevine (*Vitis vinifera* L.) is highly vulnerable to several fungal diseases, among them gray mold caused by *Botrytis cinerea* Pers.; Fr., which leads to serious damage in French vineyards, particularly in the regions where the climate is cool and humid. This fungus infects flowers, setting fruits, mature fruits, and leaves. Currently, gray mold is controlled before harvest by preventive fungicides. However, because of the increasing worldwide concern about pesticide use due to environmental problems and pathogens developing resistance, alternative plant protection strategies are becoming increasingly attractive. This has promoted the consideration of biological disease control and induction of plant resistance strategies by using either non-pathogenic plant-associated microorganisms (Van Loon et al., 1998; Bargabus et al., 2003; Tjamos et al., 2005) or components derived from microorganisms and plants (Aziz et al., 2003; Nürnberger et al., 2004).

A great number of reports indicated that certain bacterial strains are beneficial for the growth of plants; these are called plant growth-promoting rhizobacteria (PGPR). Colonization of roots with PGPR can also induce resistance in parts of the plant that are spatially separated from the inducing microorganism (Maurhofer et al., 1994; Van Loon et al., 1998). An important trait of these bacteria is their ability to maintain a stable relationship with the associated plant species (Smith and Goodman, 1999; Miethling et al., 2000). Consequently, the plant material can have a significant influence on the composition of the microflora obtained, as well as on the probability of finding isolates with biocontrol activities. Microorganisms isolated from the rhizosphere or from tissues of a specific plant are non-exotic, thereby presenting no risk of proliferation of a new microorganism in the environment. Furthermore, they may be better adapted to that plant and therefore provide better control of dis-

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eases than organisms originally isolated from other plant species (Handelsman and Stabb, 1996).

Biocontrol bacteria may protect plants against pathogens by direct antagonistic interactions between the biocontrol agent and the pathogen, as well as by induction of the host resistance. The biocontrol depends on a wide variety of traits, such as the production by the biocontrol strain of various antibiotic compounds, iron chelators and exoenzymes such as proteases, lipases, chitinases, and glucanases (Leong, 1986; Maurhofer et al., 1994; Chin-A-Woeng et al., 1998; Dunlap et al., 1998; Raaijmakers and Weller, 1998; Trejo-Estrada et al., 1998); as well as competitive root colonization (Chin-A-Woeng et al., 2000; Lugtenberg et al., 2001), and induced resistance in the host plant (Baker et al., 1985).

In recent years, considerable attention has been focused on induced resistance as an important phenomenon that occurs when plants develop enhanced defensive capacity upon appropriate elicitation. Induced defense reactions can be restricted to the tissues close to the site of elicitation or can be expressed systemically throughout the tissue or the whole plant. Bacteria-induced systemic resistance (ISR) has been demonstrated in a variety of plant species against a broad spectrum of pathogens (Hammerschmidt and Kuc, 1995; Van Loon et al., 1998; Magnin-Robert et al., 2007). In some cases, ISR is associated with the expression of some defense genes such as those encoding for pathogenesis-related (PR) proteins (e.g. chitinase) and also phenylalanine ammonia-lyase (PAL) and lipoxygenase (LOX) pathways (Maurhofer et al., 1994; Van Loon and Van Strien, 1999). LOX is required for the synthesis of the precursors of jasmonates, compounds that may act as the signal factor in plant defense responses (Creelman and Mullet, 1997; Pieterse et al., 1998). PAL is a key enzyme concerned with the synthesis of salicylic acid and phenolic compounds which were proposed to reduce incidence of plant disease through antifungal activity and stimulation of plant defense responses (Lee et al., 1995; Reymond and Farmer, 1998; Shadle et al., 2003). The relative importance of all these mechanisms differs considerably among strains of biocontrol bacteria (Neiendam-Nielson et al., 1998; Van Loon et al., 1998).

In grapevine, much of research reported on the use of the fungi *Trichoderma* spp. and *Gliocladium* spp. to control gray mold (Elmer and Reglinski, 2006). Nevertheless, a possible control of this disease by a *Burkholderia* sp. originally isolated from onion has been reported and attributed to a systemic spread of the bacterium into the aerial parts of the plant (Compant et al., 2005). Recently, a commercial biofungicide Serenade, which contains a *Bacillus subtilis* strain (QST 713), was reported to be effective against various pathogenic fungi (http://www.agraquest.com).

Our goals were to: (1) screen, identify, and characterize non-pathogenic bacteria isolated from the rhizosphere and tissues of healthy grapevine plants for their effectiveness to control gray mold on grapevine leaves caused by a highly virulent *B. cinerea* isolate and (2) quantify elicitation of some defense-related responses in grapevine leaves by selected bacterial strains.

2. Materials and methods

2.1. Isolation of bacterial strains

Bacteria were isolated during the growing season from the rhizosphere, roots, leaves and stems of healthy grapevine plants (*V. vinifera* L., cv Chardonnay) from a vineyard located in the Champagne area (Marne, France). Leaves, root and stem sections were surface disinfected (20 s with 70% ethanol, and then for 10 min in a 2% sodium hypochlorite solution for root and stem sections or for 20 s in a 2% sodium hypochlorite solution for leaves) and severely washed with sterile aqueous NaCl (0.85%). Each sample was dissected aseptically into small segments and macerated in the 0.85% NaCl solution. The rhizospheric soil samples were directly suspended in the sterile NaCl solution. Tissue and soil extracts were then serially diluted and plated in triplicate onto King's B-agar, glycerol–arginine–agar and Luria–Bertani-agar (LB-agar) media to recover bacteria present in the plant tissues and soil. Bacteria were grown on plates at 30 °C for 24–72 h. Colonies were then counted and isolated on LB-agar, cultured in LB at 30 °C for 24 h, and stored in sterile 20% glycerol solution at −80 °C.

2.2. Plant material

Grapevine plantlets (*V. vinifera* L. cv Chardonnay, clone 7535) grown in vitro from nodal explants on modified Murashige and Skoog (1962) medium (half concentration of macro-elements and glutamine at 200 mg/l), supplemented with 20 g/l sucrose, and 7 g/l agar. Plants were grown in 25-mm test tubes under white fluorescent lamps (60 μmol/m^2/s), 16/8 h photoperiod, and 25 °C day/night temperature.

2.3. Fungal pathogen

A virulent *B. cinerea* (strain 630), isolated from a vineyard in the Marne Valley (France) was a gift of Dr. Y. Brygoo (INRA, Versailles, France). It was cultured in Petri dishes on potato dextrose agar (PDA) medium (Sigma, St Quentin Fallavier, France) at 22 °C for 14 days. Conidial suspension was obtained by flooding the fungal culture with sterile distilled water, rubbing the mycelium and filtering through a sterile nylon gaze (mesh of 200 μm). The conidial suspension was adjusted with sterile distilled water to 2.5×10^5 conidia/ml.

2.4. Effectiveness of bacteria to control gray mold on grapevine leaves

Grapevine leaves excised from in vitro-grown plantlets (10-week-old) were floated with abaxial side down on the buffer surface (2 mM MES pH 5.9, containing 0.5 mM $CaCl_2$ and 0.5 mM K_2SO_4), in the presence of each bacterial isolate at 1×10^7 CFU/ml. Control consisted of leaves incubated on buffer alone. After 20 h, the leaves were rinsed with sterile distilled water, patted dry and placed in Petri dishes, the adaxial side facing a wet absorbing paper. One needle-prick wound was applied to each leaf, and the fresh wounds were covered with 5-μl drops