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Enhanced control of postharvest citrus fruit decay by means of the combined use of compatible biocontrol agents

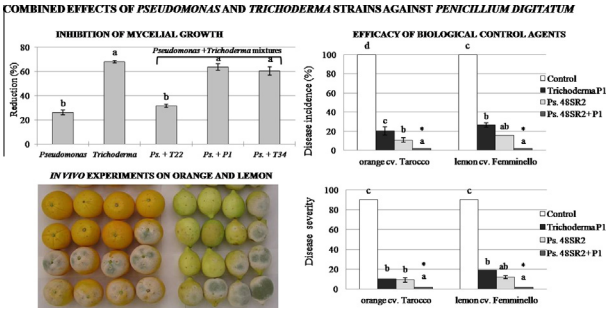
Salvina Panebianco [a,1], Alessandro Vitale [a,1], Giancarlo Polizzi [a], Felice Scala [b], Gabriella Cirvilleri [a,*]

[a] Dipartimento di Agricoltura, Alimentazione e Ambiente, Università di Catania, via S. Sofia n. 100, 95123 Catania, Italy
[b] Dipartimento di Agraria, Università di Napoli Federico II, via Università n. 100, 80055 Portici (NA), Italy

HIGHLIGHTS

- *Pseudomonas* and *Trichoderma* strains have a biocontrol activity against *Penicillium digitatum*.
- Combined use of biocontrol agents increases efficacy of biocontrol strategy.
- Synergistic effects of the mixtures are evidenced in *in vivo* conditions.
- Synergistic and antagonistic interactions in the mixture must be considered.

GRAPHICAL ABSTRACT



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ABSTRACT

Fourteen *Pseudomonas* spp. and three *Trichoderma* spp. strains were tested *in vitro* for their antagonistic properties against *Penicillium digitatum*, the causal agent of citrus green mold. Bacterial and fungal strains strongly inhibited the pathogen growth *in vitro* on PDA and on a citrus-based medium. Inhibitory effects were increased when *Pseudomonas* and *Trichoderma* strains were applied in combination. Inhibitory effects were also increased by mixtures of *Pseudomonas* cells and *Trichoderma atroviride* P1 culture filtrates. On the opposite, bacterial and fungal filtrates in mixture never induced an improved efficacy, thus indicating that the presence of living bacterial cells was required for a synergistic effect. Accordingly, incidence and severity of disease on orange cv. Tarocco and lemon cv. Femminello were consistently reduced when *Pseudomonas* and *Trichoderma* strains were applied 72 h before challenging *P. digitatum*. *Pseudomonas syringae* strains were the most effective and several combinations had a biocontrol activity higher than one of the each antagonistic microorganism applied alone. The treatments comprising six *P. syringae* in mixture with *Trichoderma* strains T22, P1 and T34 were the most effective, with 80–100% control of green mold. The development of green mold decay was also effectively inhibited in small-scale dip-treatments, either by single applications or by their mixtures. These experiments suggest that the combination of *Pseudomonas* spp. and *Trichoderma* spp. strains could be considered as a promising mean for the control of citrus green mold decay, supporting the concept that an improved disease control is given by the combined action of the two agents.

1. Introduction

Citrus is one of the most important crops cultivated in Italy and the main citrus growing areas are located in Sicily. Recently, the European Community (EC) has identified typical citrus production

* Corresponding author. Fax: +39 95 7147283.
E-mail address: gcirvil@unict.it (G. Cirvilleri).
[1] Panebianco S. and Vitale A. contributed equally to this paper.

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districts with the labels PGI (Protected Geographical Indications) and PDO (Protected Designations of Origin). The labeled products are 'Tarocco' pigmented (blood) orange and 'Femminello' lemon (PGIs, cultivated in Catania and Syracuse provinces, respectively) and 'Ribera' blond orange (PDO, cultivated in Agrigento province). The pigmented orange is a sweet orange variety typical of the eastern Sicily, which has the most suitable soil and climate conditions to produce fruits of high quality.

Postharvest green mold of citrus caused by *Penicillium digitatum* (Pers.) Sacc. is responsible for serious economic losses during harvest, transportation and storage, thus limiting the commercial life of harvested citrus fruit. The different susceptibility of citrus fruit to *P. digitatum*, more prevalent as fruit increases in maturity and at favorable temperature and humidity conditions, as well as the injuries sustained by the fruit during harvest and in the postharvest environments, can influence incidence and severity of decay. In addition, the high number of dry air-borne spores produced by *P. digitatum* not only represents an important source of inoculum in the field and particularly during storage and transportation but can also cause allergic responses (Moss, 2008).

Currently, in Italy, synthetic fungicides containing the active ingredients imazalil and thiabendazole are the primary means for the control of postharvest citrus diseases including green mold decay. However, the occurring of resistant fungal strains to many fungicides (Holmes and Eckert, 1999), the cost of developing new pesticides, their increasingly restricted use and the growing public concern over pesticide contamination of food and environment have prompted the search for alternative and safer biological control strategies.

Several means, such as natural compounds, organic and inorganic salts and antagonistic microorganisms represent the approaches recently evaluated to ensure fruit quality and safety (Sharma et al., 2009; Youssef et al., 2012, 2014; Fallanaj et al., 2013; Panebianco et al., 2014; Sanzani et al., 2014; Parafati et al., 2015). Application of bacterial strains to control postharvest decay of citrus has been extensively studied and several strains of *Bacillus*, *Burkholderia*, *Pantoea*, *Pseudomonas* and *Serratia* spp. able to reduce disease caused by the most common postharvest citrus pathogens were found (Cirvilleri et al., 2005; Meziane et al., 2006; Cañamás et al., 2008; Scuderi et al., 2009; Hao et al., 2011). However, only strains of *Pseudomonas syringae* have been mass-produced and commercialized under the name of BioSave to control postharvest citrus pathogens. Although the modes of action of bacterial biological control agents (BCAs) of postharvest diseases are not completely understood, competition for space and nutrients, production of siderophores, induction of defense responses and antibiotic production appear to be involved in many mechanisms (Sharma et al., 2009; Nunes, 2012).

Other microorganisms that have a potential to protect citrus fruit from disease are the fungi of the genus *Trichoderma*. They are used against soil-borne fungi such as *Calonectria*, *Fusarium*, *Pythium*, *Rhizoctonia* and *Sclerotinia* (Fravel, 2005; Vitale et al., 2012a), but also to control postharvest pathogens, including *Aspergillus* spp., *Botrytis cinerea*, *Monilia fructigena*, *P. expansum*, *Rhizopus stolonifer* on apples, grapes, peaches, pears and strawberries (Hong et al., 1998; Batta, 2007; Senthil et al., 2011; Vitale et al., 2012b), *Colletotrichum gloeosporioides* and *Botryodiplodia theobromae* on rambutan fruits (Sivakumar et al., 2000) and *P. digitatum* on orange fruits (Borrás and Aguilar, 1990). *Trichoderma* spp. have been reported to compete for nutrients, parasitize fungal pathogens, induce resistance in the host plant, produce cell wall-degrading enzymes (CWDEs) and antibiotics (Lorito et al., 1993; Howell, 2003; Marra et al., 2006).

Several studies have demonstrated that efficacy in biocontrol of postharvest pathogens can be increased by the use of combinations of BCAs or their metabolites. Mixtures of complementary and non-competitive BCAs may have a wider spectrum of activity (different fruits and cultivars) and can control more than one disease simultaneously. Efficacy of BCA mixtures could result not only from activity of single species but also from their synergistic action that can suppress a target pathogen through different mechanisms of action (Fogliano et al., 2002; Woo et al., 2002). Furthermore, in a mixture, the presence of an antagonist could improve the efficacy of the other biocontrol agent. For example, bacterial biocontrol agents could utilize the nutrients released by chitinolytic enzymes from hyphae of target fungi, and the subsequent increase in bacterial populations should enhance their ability to act as biocontrol agents (Lorito et al., 1996). Accordingly, the combination of cyclic lipodepsipeptides (LDPs) produced by *P. syringae* and cell wall degrading enzymes from *Trichoderma* strains was found to synergistically enhance their antifungal activity if compared with relative single applications (Lorito et al., 1996; Fogliano et al., 2002). The application of BCA mixtures improved the efficacy of biocontrol in many agricultural systems, some of which also include pathogens that infected fruits (Guetsky et al., 2001; Meziane et al., 2006; Calvo et al., 2010).

To our knowledge, no study has been conducted to determine the ability of mixtures of *P. syringae* and *Trichoderma* spp. to improve their biocontrol activity against *P. digitatum* on orange and lemon. Therefore, the main aim of this research was to select strains of *Pseudomonas* and *Trichoderma* with a putative high potential for biological control activity and to examine potential benefits of combining various strains or their metabolites. In addition, the efficacy of the selected biocontrol agents and their mixtures in controlling citrus green mold was evaluated as preventive application by dip-treatment.

2. Materials and methods

2.1. Bacterial and fungal strains

Fourteen *Pseudomonas* spp. strains and three *Trichoderma* spp. strains were used in screening tests. Bacterial strains (*P. syringae* 281_1, 287_1, 291_1, 335_2, 40SR4, 48SR2, B728a, 46P, 1.3S and HRI1480A; *Pseudomonas fluorescens* P17D and Z4.9; *P. corrugata* G6E and G9C), previously isolated and characterized (Cirvilleri et al., 2005; Dimartino et al., 2011), were maintained in 15% glycerol at −80 °C and subcultured on nutrient agar medium (NA, Oxoid) or King's medium B (KB) (King et al., 1954) as needed.

Isolates of *Trichoderma harzianum* T22, *Trichoderma atroviride* P1, and *Trichoderma reesei* T34 were kindly provided by prof. Matteo Lorito, Department of Agriculture, University of Naples. *P. digitatum* (Sacc. was isolated from an infected fruit, characterized (Oliveri et al., 2007), and maintained on potato dextrose agar (PDA, Oxoid, Basingstoke, UK).

2.2. In vitro inhibitory activity of Pseudomonas and Trichoderma alone and in mixture

Tests were carried out on PDA and on orange-peel-extract-agar (OPEA) (100 g l^{-1} of orange peel extract; 20 g l^{-1} of agar) to evaluate inhibition activity of the individual antagonist strains and their 1:1 mixtures against *P. digitatum.*

Aliquots of 20 µl of bacterial suspensions (10^9 CFU ml^{-1}) were spotted on PDA and OPEA plates (one spot per plate) and 20 µl aliquots of conidial suspensions (1×10^6 CFU ml^{-1}) of *P. digitatum* spotted 3 cm away from the bacterial spot. After incubation at 27 °C for 7 days, reduction of mycelial growth (%) of *P. digitatum* compared to the control was calculated.

The antagonistic activity of *Trichoderma* spp. strains against *P. digitatum* was carried out according to Sivakumar et al. (2000) with