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Isolation of bio-protective microbial agents from eco-composts

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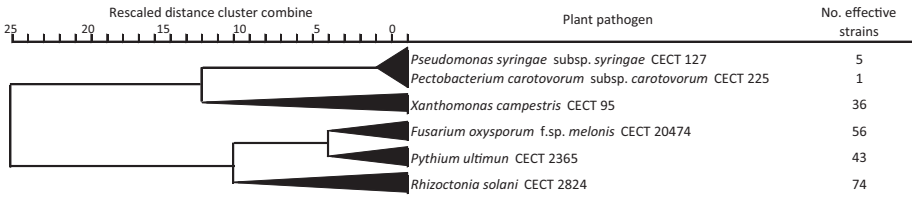
HIGHLIGHTS

- Selection of some broad spectrum biocontrol agents from plant-based compost.
- Two strains identified as *B. subtilis* and *P. chrysogenum* were effective against Fom.
- The best biocontrol agent showed a disease reduction range near to 50%.
- The best biocontrol agent weakly affected plant health in the absence of phytopathogen.

GRAPHICAL ABSTRACT



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ABSTRACT

Although increasing soil fertility is the main use of compost, the presence of bio-protective microorganisms against plant pathogens confers it an added value.

Here we review a microbial collection from plant waste based composting piles, and we tested the selected microbiota towards phytopathogenic bacteria and fungi. The raw material used for composting mixtures was vegetable waste from organic agriculture. Compost samples were collected at different stages of the composting process and one hundred and twenty-six microbial strains were selected. Phytopahtogenic strains were supplied by the Spanish Type Culture Collection: *Fusarium oxysporum* f.sp. *melonis* CECT 20474, *Rhizoctonia solani* CECT 2824, *Pythium ultimum* CECT 2365, *Pectobacterium carotovorum* subsp. *carotovorum* CECT 225, *Pseudomonas syringae* subsp. *syringae* CECT 127 and *Xanthomonas campestris* CECT 95.

Forty out of all tested isolates showed *in vitro* antagonistic activity against at least three out of the six phytopathogenic agents investigated. Six strains were then selected and *in vivo* tested to induce systemic resistance in melon plants towards the fungus *Fom.* In the presence on antagonistic strains, plants exhibited an enhanced defensive capacity against the pathogenic fungus as compared with non-inoculated control plants. Two strains identified as *Bacillus subtilis and Penicillium chrysogenum* showed a higher antagonistic capacity against Fom. These biocontrol agents showed a disease reduction range near to 50% and weakly affected plant health in the absence of phytopathogen.

On the basis of the results here shown, this study was successful in selecting some biocontrol agents which showed to be effective against important and devastating phytopathogen microorganisms. According to this research work, these microorganisms could potentially be formulated and used as biopesticide products, avoiding the adverse environmental effects of chemical hazardous pesticides.

1. Introduction

Several studies have shown the suppression of numerous plant diseases by the use of organic amendments from heterogenic sources such as agro-industrial wastes including fresh plant, grape or winery wastes, manure, rubbish and sludge (Ntougias et al., 2008; Suárez-Estrella et al., 2007) however, this suppressive capacity is highly variable, depending on composted materials, application doses or compost aging (Bonanomi et al., 2007; Noble and Coventry, 2005).

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The production of fungitoxic compounds such as organic acids or ammonia from some organic amendments contributes to the observed antagonistic effect (Bollen et al., 1989). In addition, the presence of typical microorganisms in these substrates could enhance the antagonistic mechanisms involved in plant pathogen suppression. In fact, suppressive capacity of composts has been attributed to complex interactions between abiotic and biotic factors (Pérez-Piqueres et al., 2006; Van Loon, 2007). On the other hand, the use of compost in agriculture and horticulture as a natural nutrient source can contribute to waste recycling and reduce the use of other more harmful (chemically-synthesized) fertilizers.

Fusarium oxysporum f.sp. *melonis* (Fom) is one of the most harmful phytopathogenic agents detected along the Mediterranean coast. The mild climatic conditions favor the occurrence of this phytopathogen (González et al., 1988; Tamietti and Valentino, 2006) so its suppression is now considered an urgent need for Mediterranean agriculture. Currently, chemical methods used to control vascular wilt caused by Fom are inefficient or imply environmental damage, both of which are detrimental to human health. In this sense, alternatives to chemical pest control are being extensively investigated (Pascual et al., 2002; Suárez-Estrella et al., 2007, 2012).

In contrast to the general suppression of phytopathogenic oomycetes that seems to be related to the proliferation and activity of broad microbial consortia (Hoitink et al., 1996), specific microbial agents appear to be responsible for the efficiency of compost amendments on the control of eumycetes such as *Fusarium oxysporum* and *Rhizoctonia solani* (Ntougias et al., 2008).

Various studies related to biological control of Fusarium wilt by suppressive compost or by antagonistic microorganisms from compost have helped to diminish the incidence of these diseases around the world (Kavroulakis et al., 2005; Suárez-Estrella et al., 2007). In this sense, a disease reduction up to 90% has been achieved in several cases.

The main objective of this work was the isolation and identification of novel, native and effective broad-spectrum biocontrol agents from eco-compost. This objective was achieved by evaluating their *in vitro* antagonistic activity against major bacterial and fungal soilborne and foliar pathogens and their effectiveness to suppress *in vivo* the melon plant pathogen *Fusarium oxysporum* f.sp. *melonis*.

2. Material and methods

2.1. Plant pathogens

Phytopathogenic strains were supplied by the Spanish Type Culture Collection (CECT). Bacterial cultures of *Pectobacterium carotovorum* subsp. *carotovorum* CECT 225 (Pcc), *Pseudomonas syringae* subsp. *syringae* CECT 127 (Pss) and *Xanthomonas campestris* CECT 95 (Xc), were kept in slants on nutrient agar (NA; CM0003, Oxoid Ltd. UK) at 4 °C, while fungal cultures of *Fusarium oxysporum* f.sp. *melonis* CECT 20474 (Fom), *Rhizoctonia solani* CECT 2824 (Rs) and *Pythium ultimum* CECT 2365 (Pu) were kept on potato dextrose agar (PDA; CM0139B, Oxoid Ltd. UK) at 4 °C.

2.2. Compost material used

Three composting piles (3 m × 1.5 m × 1 m) were made of various vegetable materials to be used as the source of strains. The raw materials for the mixtures were pea and cucumber plant waste and pruning waste in a proportion of 50:25:25 (by volume). Plant material came from organic agriculture facilities. Aeration and moisture were controlled. Piles were periodically aerated by turning (approximately every 15 days) while there were fluctuations in temperature. During turning operations, water was added when necessary. The whole process including maturation lasted for 150 days.

2.3. Isolation of potential antagonistic microorganisms

Compost samples were collected at different stages of the composting process. Samples were taken at 0, 14, 28, 45, 73, 100 and 150 days after the beginning of the process. Actinobacteria were recovered using Sodium Caseinate Agar (SCA: sodium caseinate, 0.20 g; K_2HPO_4, 0.50 g; $MgSO_4$, 0.20 g; $FeCl_3$, 0.01 g; agar, 16 g; distilled water, 1 L; pH 6.5) and incubated at 30 °C for 72 h. Bacteria were isolated using Nutrient Agar (NA) plates and incubated at 30 °C for 24 h. In both cases, representative colonies growing on plates were selected, isolated and kept in NA at 4 °C. Fungal strains were isolated using Rose-Bengal Chloramphenicol agar plates (RB CM0549B, Oxoid Ltd. UK) and incubated at 30 °C for 96 h. In this case, representative colonies growing on plates were selected, isolated and kept in PDA at 4 °C.

The final strain collection consisted of 35 actinobacterial, 46 bacterial and 45 fungal strains. The different strains selected were named with a code indicating the compost pile source (PI, PII or PIII), the sampling time (T0–T150) and the microbial type (B, A or F: Bacterium, Actinobacterium or Fungus).

2.4. In vitro evaluation of antagonistic activity against plant pathogens

2.4.1. Antagonistic activity of isolated bacteria (AB) and actinobacteria (AA) against phytopathogenic bacteria (PB)

Each potentially antagonistic bacterial and actinobacterial strain was cultured on nutrient broth (NB; CM0001, Oxoid Ltd. UK) for 24 and 96 h respectively at 30 °C prior to use. Suppressive effect of cultures was demonstrated using a slightly modified version of the technique described by de Boer et al. (1999). First, 2% water agar (WA) plates were prepared and, after its solidification, two 8-mm-diameter steel hollow cylinders were placed equidistantly from the edge of the plate. A second layer of NA was added on the WA plates. Once NA had solidified, the cylinders were removed and two empty wells were obtained. A PB (Xc, Pss or Pcc) liquid culture was streaked on the plate surface with a sterile swab. The wells were then filled with 50 ml of the antagonist liquid culture to be assayed and the plates were incubated at 30 °C for 48 h. The plates were observed for clear (inhibition) zones around the wells. Two replicated plates were used for each antagonist-PB combination. Inhibition index (I) was expressed as percentage of PB growth inhibition in the presence of the antagonistic strain.

2.4.2. Antagonistic activity of isolated bacteria (AB) and actinobacteria (AA) against phytopathogenic fungi (PF)

Potentially antagonistic cultures were prepared as described previously. In this case, suppressive effect was demonstrated using the modified techniques of Landa et al. (1997). First, 2% water agar (WA) plates were prepared. After the agar was solidified four 8-mm-diameter steel cylinders were placed equidistantly from the edge. A second layer of PDA was added on the WA plates. Once the cylinders were removed, the wells were filled with 50 ml liquid cultures of the antagonist to be assayed and a plug of 5-days-old PF (Fom, Rs or Pu) culture was removed from a PDA plate and placed at the center of the assay plate. There were two replicated plates for each antagonist-PF combination. Plates were incubated at 30 °C for 5 days and the inhibition index (I) was expressed as percentage of PF growth inhibition in the presence of the antagonistic strain.

2.4.3. Antagonistic activity of isolated fungi (AF) against phytopathogenic bacteria (PB)

Suppressive effect of fungal cultures was demonstrated using the modified technique of de Boer et al. (1999). A PB (Xc, Pss or