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Mutualistic interaction of rhizobacteria with arbuscular mycorrhizal fungi and its antagonistic effect on *Fusarium oxysporum* in *Carica papaya* seedlings

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ABSTRACT

There are numerous studies evaluating biocontrol of root rot by using the antagonistic effects of either arbuscular mycorrhizal fungi (AMF) or rhizobacteria, but usually independently. Fewer studies, although growing in number, report on evaluating the effectiveness of concurrent fungi–bacteria inoculation in combating root rot; and furthermore, there are none to date reported with papaya. In this study, an indigenous *Pseudomonas* sp. (PPV3) was isolated from roots of papaya (*Carica papaya* L. cv. Maradol) and used with an AMF complex (MTZ01) consisting of four fungi *Glomus intraradices*, *Glomus mosseae*, *Glomus etunicatum* and *Gigaspora albida* to inoculate roots of papaya in order to determine their antagonistic effects against *Fusarium oxysporum*, individually and in combination. It was found that with inoculation with PPV3 and MTZ01 protection was highest (85%) and had reduced disease (10%) as well as reducing *F. oxysporum* colonization in papaya seedlings. Inoculations with MTZ01 or PPV3 showed an efficacy of 54 and 60%, with a level of disease severity of the 38 and 22%, respectively. The combination of the AMF complex (MTZ01) with rhizobacterial *Pseudomonas* sp. (PPV3) modified the effects of *F. oxysporum* and provided increased protection for *C. papaya* than either acting alone. These results suggest that rhizobacteria and arbuscular mycorrhizal fungi acting together formed a mutualistic relationship that enhances disease control against *F. oxysporum* and stimulates growth in *C. papaya*.

1. Introduction

In agroecosystems, the maintenance of soil fertility and productivity is a crucial goal of sustainable agriculture (Bedini et al., 2007). Soil borne pathogens play a direct role causing significant economic losses in agriculture, in particular by fungi, such as *Fusarium* sp. (McMullen et al., 1997; Windels, 2000), which is one of the most aggressive (Migheli et al., 1992). Nevertheless, disease causing effects of *Fusarium* spp. have intensified over the years, attributed to changes in farming practices (Khade and Rodrigues, 2009a), climate change (Chakraborty et al., 2010) and soil depletion (Pimentel et al., 2005). Plant diseases caused by soil fungi are found in several phytopathogenic genera, such as *Phytophthora*, *Rhizoctonia* and *Fusarium*, as well as oomycete *Pythium*.

Papaya (*Carica papaya* L. cv. Maradol) is cultivated for its fruit, mainly in tropical and subtropical climates in Asia and South and Central America. In 2010, worldwide production of this fruit was slightly less than 11.2 million tonnes (Mt) on about 438,239 ha (FAO, 2010). Papaya, like many crops, is susceptible to wilting caused by the fungus *Fusarium oxysporum* (Nishijima, 1994). In papaya and other plants, *F. oxysporum* survives as chlamydospores and, when in contact with roots, will germinate and infect the root systems (Khade and Rodrigues, 2009b).

The current preferred method of control or crop protection against *Fusarium* spp. is to rotate with crops that are not hosts for up to four consecutive years and, in some regions, by applying fungicides (Koike et al., 2007).

Biological control using living organisms is a strategy for protecting crops from soil-borne plant pathogens (Guetsky et al., 2002; Silva and Bettiol, 2005; Nam, 2009). For example, arbuscular mycorrhizal fungi (AMF) are probably one of the most-researched groups of endophytic microorganisms because they enhance

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plant growth through improved nutrient uptake (Sundram et al., 2011). AMF are found in the roots of 80% of vascular plants and there is increasing evidence that the combination of bacteria–fungi interactions are more widespread than expected and may be essential in ecosystems (Bonfante and Anca, 2009). This symbiosis is based primarily on bidirectional nutrient transfer between all the symbionts, in other words, between fungi, bacteria and plant (Smith and Read, 2008).

There are many studies using *Pseudomonas* spp. to control *Fusarium* spp., such as with carnation (Van Peer and Schippers, 1992), radish (Raaijmakers et al., 1995; De Boer et al., 2003), banana (Saravanan et al., 2004) and many with tomato (Duijff et al., 1998; Ramamoorthy et al., 2002; Bolwerk et al., 2003; Manikandan et al., 2010; Hariprasad et al., 2011). In papaya, the plant of this study, there are reports of AMF fungi increasing plant resistance to pathogen biotic stresses (Sukhada et al., 2011), but none using both *Pseudomonas* sp and AMF fungi in combination.

Suppression of soil-borne pathogens by rhizosphere microorganisms has been studied for more than 65 years (Cook and Baker, 1983). As early as the 1980s, *Pseudomonas* sp. has been reported to act as a dominant microflora in the microbiological rhizosphere ecosystem (Weller, 1988). For *Pseudomonas* spp. the mechanisms for controlling pathogenic fungi are apparently from production of antimicrobial metabolites (Neilands, 1981), where siderophores are produced that play a role in iron-chelating, thereby limiting iron availability to pathogenic fungi and inhibiting growth (Ramamoorthy et al., 2001; Pal and McSpadden, 2006) by production of antifungal metabolites (Van Loon et al., 1998). *Pseudomonas* spp. are capable of producing other compounds, such as hydrogen cyanide (HCN), enzymes and phytohormones that inhibit pathogenic microorganisms (Schippers et al., 1991; Gupta et al., 2001). The *Pseudomonas* spp. are particularly known to restrict pathogens by producing metabolites with antimicrobial activity (Chen et al., 2000). Most of our knowledge of mechanisms and metabolites in biological control by bacteria are based on studies with fluorescent *Pseudomonas* spp. (Raaijmakers et al., 2002).

Recent evidence suggests that endophytic bacteria are important and play definite roles in plant host tissues and colonization, which has generated interest in using them as agricultural tool to improve protection and crop yield (Siddiqui, 2006). One of these important roles is as mycorrhizae helper-bacteria (MHB), in which the bacterial strains assist mycorrhizal formation. Several bacteria that interact positively with the function of symbiosis have been reported in reviews by Frey-Klett and Garbaye (2005) and more recently again by Frey-Klett et al. (2007).

The mutualistic relationship between fungi and bacteria affects the health and growth of plants. Also, there are many studies combining fungi and bacteria in plant hosts against plant soil-borne plant pathogens. Unlike plant pathogens, which induce disease or plant defense responses when introduced into host plants, mutualistic endophytes do not usually cause any visible reaction (hypersensitive response) or disease (Ezra et al., 2010). This is the first study carried out to determine the ability of arbuscular mycorrhizal fungus and *Pseudomonas putida* separately and in combination to influence disease outcomes in *C. papaya* seedlings caused by *F. oxysporum*.

2. Materials and methods

2.1. Pathogen fungi

F. oxysporum FOPV001 is a fungus isolated from diseased papaya cv. Maradol plants, part of the collection of microorganisms of the Laboratorio de Organismos Benéficos-Universidad Veracruzana, Campus Xalapa, Veracruz, México. The fungus was cultivated in potato dextrose broth in rotary shaker flasks of 250 ml Erlenmeyer at 25 °C for four days. The culture was filtered through a sterile, number 2 sintered glass funnel (40–100 μm pore size mesh) to retain the mycelia. The microconidia remaining in the filtrate were harvested by centrifugation (5000 × *g*; 20 min) and washed twice in sterile distilled water. The concentration of the conidia was estimated under the microscope with a hemocytometer and adjusted with sterile distilled water.

2.2. Mycorrhizal complex inoculum

The AMF complex MTZ01 were also provided by the Laboratorio de Organismos Benéficos-Universidad Veracruzana. It is composed of *Glomus intraradices*, *Glomus mosseae*, *Glomus etunicatum* and *Gigaspora albida*, originally isolated from Maradol papaya plants. The complex MTZ01 culture was maintained in pots with *Zea mays* and *Triticum sativum* as host plants. The pots were cultivated in a greenhouse with ambient natural light and temperature conditions and irrigated with de-ionized water for 12 weeks. Mycorrhizal complex inoculum was prepared by inoculating the plants with 10 g of substrate (per pot) containing 40 spores g^{-1}, together with mycelium and mycorrhizal root fragments.

2.3. Antagonistic rhizobacteria

Eight rhizobacteria wild-type strains of *Pseudomonas* sp. were selected and isolated from the rhizosphere of Maradol papaya plants maintained in King B (KB) medium (King et al., 1954) at 25 ± 2 °C and labeled PPV1, PPV2, PPV3, PPV4, PPV5, PPV6, PPV7 and PPV8. For long-term maintenance, the strains were preserved in nutrient broth containing 15% (v/v) glycerol at −70 °C.

2.4. In vitro screening of biocontrol rhizobacteria

To determine fungal growth inhibition capacity of *Pseudomonas* isolates, a 0.5 cm^2 plug of 6-day-old *F. oxysporum* culture was inoculated in the center of a 9 cm diameter Petri dish. Four sterile filter paper discs were placed at equal distance to the *F. oxysporum* disc with 10 μl rhizobacteria suspension grown in KB medium on each paper disc. The plates contained potato-dextrose-agar (PDA). For determining the antagonistic siderophore effect, 80 μM $FeCl_3$/l (Fe^+; Akköpru and Demir, 2005) was added to the medium (deficient in ferric ions, Fe^-). The plates were incubated at 25 °C for 8 days. Each isolated rhizobacterium was replicated four times and the test was repeated two times. Differences in diameters of inhibition were measured (in mm) and the antagonistic siderophore effect was determined using a scale from 0 to 5 (Geels and Schippers, 1983): with **0** = no inhibition; **1** = $x \leq 2$ mm; **2** = 2 mm $< x < y$; **3** = 2 mm $< x > y$; **4** = $y \leq 2$ mm; **5** = $y = 0$, where x is the zone where the pathogen was inhibited, y is the zone where the pathogen developed. Loss of inhibition zones in the dishes containing $FeCl_3$ was an indication of competition of for the Fe^{3+} ions (Bora and Özaktan, 1998). Growth inhibition (GI) was calculated by using the formula in Singh et al. (2002):

$$\mathrm{GI}(\%) = \frac{G_c - G_t}{G_c} \quad (1)$$

where G_c is the growth diameter in the control plate and G_t is the growth diameter in the treatment plate.

2.5. Molecular identification of Pseudomonas sp.

Rhizobacteria *Pseudomonas* strain PPV3 showed the higher antagonistic activity *in vitro* and was selected for molecular