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Biological Control of Phytopathogenic Fungi by Endophytic Actinomycetes Isolated from Maize (*Zea mays* L.)

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ABSTRACT

This work aimed a survey on the biodiversity of maize endophytic actinomycete, and an evaluation of their potential to control the phytopathogenic fungi. From several regions of São Paulo state, 40 strains were isolated from the healthy maize plants. The identification of these strains, based on morphological properties and fatty acid methyl ester (FAME) profile showed that most of them belonged to the Streptomyces *genus. These isolates were first screened for the growth inhibition of phytopathogenic fungi and results showed that all the isolate were able to inhibit the development of at least one tested pathogen. Two selected isolates were then evaluated for the control of* P. aphanidermatum *in cucumber (*Cucumis sativa *L.) under greenhouse conditions. Isolate 16R3B was able to reduce up to 71% damping-off incidence whereas isolate 14F1D/2 reduced the disease incidence by 36%. Damping-off control in cucumber, mainly for the isolate 16R3B, suggested for its use in greenhouse cucumber producing fields and to be tested in field trials.*

Key words: Endophytes, actinomycete, *Streptomyces*, *Pythium aphanidermatum*, dumping-off

INTRODUCTION

Currently, there is an increasing public concern regarding the continued use of agrichemicals to control the phytopathogenic fungi. This awareness relies mainly in the noxious effects of the pesticides on the environmental and human health (Cardoso et al. 2010). Several efforts have been made to find less hazardous options for controlling these plant pathogens among which the biological control using the microorganisms has been demonstrated to be a feasible alternative (Zucchi et al. 2008) but it is not widely used on commercial scale (Bressan 2003; Medeiros et al. 2012).

Among the biocontrol agents, endophytic microorganisms have raised special attention, mainly due to their crucial role on host-plant development (Firáková et al. 2007). Since these symbionts are systemically distributed in the plant via metabolic translocation (colonizing the same niche of the phytopathogens), they are interesting candidates for the biological control (Rai et al. 2007). Bacterial endophytes have been demonstrated to inhibit the plant pathogen development as well as to promote the growth of host plants (Hasegawa et al. 2006). A deeper understanding of the endophyte-host plant interaction can enhance the use of these microorganisms in the agriculture (Araújo et al. 2000; Lee et al. 2008). Currently, endophytic bacteria have been isolated from a huge variety of plant species but only a few crop species have been completely studied related to their endophytic community (Ryan et al. 2008). In maize, several reports have demonstrated its association with the endophytic bacteria (mainly *Pseudomonas*, *Enterobacter* and *Bacillus*) (Fisher et al. 1992; Mcinroy and Kloepper 1995;

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Lodewyckx et al. 2002; Rai et al. 2007); however, only a few works have been made to isolate the endophytic actinomycete of this important crop.

Actinomycetes have been largely exploited mainly because their capability to produce bioactive compounds, such as antibiotics and lytic enzymes (El-Tarabily et al. 1997; Bérdy 2005; Clardy et al. 2006). In fact, antibiotics produced by the filamentous bacteria, mainly *Streptomyces* spp., have been reported to be able to inhibit the development of a broad range of phytopathogenic fungi and/or bacteria (Berg et al. 2001). Also, these compounds have often been related as one of the most important tools to control the soil-borne diseases (Buchenauer 1998) with low environmental impact and toxic effect for humans and animals, well-desired traits for new consumer's requirements (Cardoso et al. 2010).

One of these important soil-borne diseases is the root rot caused by *Pythium aphanidermatum*. This cosmopolitan pathogen can infect a huge variety of hosts, leading to severe economic losses, including monocots like maize and dicots cucumber (Postma et al. 2000; Zhang and Yang 2000; Veit et al. 2001). The control of *P. aphanidermatum* is usually difficult due to the lack of resistant cultivars and registered fungicides, which are only effective if used as preventive application (Postma et al. 2000). Root rot is generally a severe problem in high temperature and humidity regions, or in the greenhouse conditions. Due to its polyphagic nature, *P. aphanidermatum* can be used in screening programs as a reliable tool for enhancing the chance of selecting biocontrol agent for use in multiple crop systems. Thus, this work focussed on identifying the actinomycete endophytically-associated with maize (*Zea mays* L.) by using the culture-based approach to determine the ecological role of such interaction. Furthermore, the potential to control *P. aphanidermatum* using these endophytes were evaluated in cucumber seedlings under greenhouse conditions.

MATERIALS AND METHODS

Plant Material

Plant tissues (roots, leaves, stems) of maize (*Zea mays* L.) were collected from the rural area in four cities (Lins, Arthur Nogueira, Ouroeste and Salto Grande) in the São Paulo State, Brazil. All the areas had cultivated the same hybrid maize and the plants collected were around 60 days-old. The samples consisted of tree maize plants chosen randomly from the crop field.

Isolation of Endophytic Actinobacteria Strains

Plant tissues (leaves, stems and roots) were water washed to remove the soil residues and dust, and thereafter chopped in pieces of 8-12 cm. All the samples were surface disinfected following Zucchi et al. (2008), then exposed to UV light for 10 min, cut in smaller pieces and placed on the plates containing starch-casein-Agar medium (SCA; Küster and Williams, 1964) amended with nistatin (50 μg/mL). Inoculated plates were incubated at 28ºC and actinobacterial growth was assessed at the intervals of 5 days until the 20th day. The colonies were purified in Potato-Dextrose Agar (PDA; Beever and Bollard 1970) and stored at 4^{o}C.

Actinobacteria Strains Characterization

Each isolated strain was identified based on the whole-cell cellular fatty acids, derivatized to methyl esters (FAME) method (Sasser 1990) and analysed by a Hewllet Packard gas chromatograph model fitted with a fused silica capillary column (25 m x 0.2 mm internal diameter). The interface was obtained by the *ChemStation* A.09.01 [1206] and MIDI *Microbial Identification System 4.0* (Sherlock TSBA Library, MIDI ID, Inc., Newark, ED, USA) was used for phylogenetic analysis. The isolates with similarities indices (SIM) ≥ 0.3 were considered positively identified, whereas the isolates with a SIM < 0.3 were considered tentatively identified (Siciliano and Germida 1999; Misko and Germida 2002).

Actinobacteria and Phytopathogenic Fungi Interaction

This analysis was performed using five phytopathogenic fungi – *Fusarium* sp., *Pythium aphanidermatum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Phytophthora parasitica* – from the stock collection of the "Laboratório de Microbiologia Ambiental" at EMBRAPA, Jaguariúna, São Paulo State, Brazil. The antagonism assay with actinobacteria strains and phytopathogens were carried out in Petri dishes containing PDA medium. First, the actinobacterium was inoculated near the edge of the PDA plate. After that, a disc with 5 mm of diameter (removed from the edge of

phytopathogenic colony) was placed at 3 cm distance from the actinobacterium inoculation point. The plates were incubated at 28°C for 5-7 days.

Actinobacteria Secondary Metabolites Extraction and Antibiosis Analysis

The actinobacteria strains were inoculated in Potato-Dextrose (PD) medium and incubated in shaker (150 rpm) at 28°C for seven days. The extractions of secondary metabolites were performed according to Canova et al. (2010) using ethyl acetate as solvent. The extracts were diluted to a final concentration of 20 $\mu g.\mu L^{-1}$ and they were stored at 4°C. An antibiosis assay was performed similarly as the antagonism assay. A total of 200 µg of metabolite extract (20 $\mu g.\mu L^{-1}$) was applied over a filter paper of 5 mm of diameter, placed on a Petri dish containing the PDA medium. A disc with 5 mm of diameter containing the phytopathogenic fungus was placed at 3 cm distance from the filter paper. The plates were incubated at 28°C for 5-7 days.

Chitinolytic Analysis

Isolates 16R3B and 14F1D/2 were inoculated in tryptic-soy broth (TSB), supplemented with 0.5% chitin and incubated under the constant agitation at 28°C for five days. Chitinolytic analysis was carried out following the CM-Chitin-RBV (Loewe) manufacturer protocol. Briefly, the cultures were centrifuged (4°C, 14,000 xg, 5 min) and 25 µL of supernatant was incubated using 25 µL of CM-Chitin-RBV (Loewe), 50 L of Tris-HCl buffer (100 mM, pH 7.5) at 45°C for 2h. The reaction was interrupted using 50 µL of HCl 2N. The reading was performed using a wavelength of 550 nm and the enzyme was quantified following the method of Guzzo and Martins (1996). One unit (U) of the enzyme activity was defined by absorbance (abs) variation in one ml of substrate per min ($abs.ml^{-1}.min^{-1}$).

Biological Control of *Pythium aphanidermatum* in Cucumber Seedlings

The biological control of *P. aphanidermatum* was evaluated under greenhouse conditions. Seeds of cucumber were sowed in the substrate containing a mixture of sterilized soil and vermiculite (1:2). After 20 days, the seedlings were removed from the substrate and treated with the antagonists. The treated-seedlings were immersed (only the root) for 30 min in an antagonist suspension containing 10^8 $cfu.ml^{-1}$. The control was treated with the sterilized water. In the phytopathogen treatments, the soil was infested with *P. aphanidermatum* keeping a proportion of 200 g of inoculum per 12 L of soil. After soil infection with the pathogen, an interval of 24h was given before planting the seedlings. After these procedures, the seedlings were transplanted in 1 L pots and the treatments (T) used were: T1: Negative control; T2: Positive control (phytopathogen inoculation); T3: Fungicide control (3 g/L of Metalaxyl – APRON 35PM); T4: 14F1D/2 strain + *P. aphanidermatum* and T5: 16R3B strain + *P. aphanidermatum*. Each treatment consisted of four repetitions with seven seedlings. After 14 days of the transplanting, the incidence of disease (damping-off) was evaluated for each treatment. This experiment was conducted twice. The data were subjected to a Tukey-test ($p<0.05$), using the Sigma Stat program (Jandel Scientific, San Rafael, CA).

RESULTS

Isolation and Characterization of Endophytic Actinobacteria

A total of 40 endophytic strains were isolated from the maize plants, which included 20 from the leaves and 10 each from the stems and roots (Table 1). These isolates had morphological properties consistent to their classification in the order *Actinomycetales* and therefore, they were submitted to FAME analysis for the identification using a threshold of 30% similarity as exclusion criteria (Siciliano and Germida 1999; Misko and Germida 2002). Most of isolates were grouped within the *Streptomyces* genus. However, six strains (12R5/A, 21F2A, 8F2, 19C3D/B, 19R2C and 18F1D/A) demonstrated a low similarity index (SIM<0.3) with *Streptomyces* genus and other six strains (18C1A/B, 14R3, 21C3A1, 18F3B, 18C2D and 19C3B/D) were not effectively identified by FAME analysis.

Table 1 – Endophytic actinobacteria community isolated from maize plant (positively or tentatively) identified by FAME analysis (Sherlock TSBA Library Version 4.0).

Isolation Source	Strain	Identification [a]	Similarity Index [b]
LEAF	**18F2C**	*Streptomyces albidoflavus*	45.5%
	18F2A	*Streptomyces anulatus*	30.1%
	14F1C/B	*Streptomyces californicus*	34.2%
	18F2G	*Streptomyces halstedii*	56.8%
	11F2A'	*Streptomyces halstedii*	53.8%
	15F3B	*Streptomyces halstedii*	48.1%
	11F2A	*Streptomyces halstedii*	44.4%
	P4F1	*Streptomyces halstedii*	42.2%
	19F2C/1	*Streptomyces halstedii*	41.7%
	20F3F	*Streptomyces halstedii*	41.6%
	19F2C/A	*Streptomyces lavendulae*	57.3%
	18F3G/1	*Streptomyces lavendulae*	56.3%
	16F3B	*Streptomyces lavendulae*	53.6%
	19F1C/2	*Streptomyces lavendulae*	46.4%
	14F1D/2	*Streptomyces violaceusniger*	68.3%
	14F1D/1	*Streptomyces violaceusniger*	50.2%
	18F1D/A	*Streptomyces* sp.	25.8%
	8F2	*Streptomyces* sp.	23.0%
	21F2A	*Streptomyces* sp.	2.5%
STEM	**20C1A/B**	*Streptomyces californicus*	34.4%
	13C1B'	*Streptomyces halstedii*	63.8%
	13C1B	*Streptomyces halstedii*	54.5%
	19C3B/C	*Streptomyces lavendulae*	70.8%
	18C1C/B	*Streptomyces lavendulae*	65.8%
	19C3D/B	*Streptomyces* sp.	25.4%
ROOT	**14R2K**	*Streptomyces exfoliatus*	42.6%
	8R20	*Streptomyces glaucescens*	41.0%
	9R1	*Streptomyces halstedii*	44.0%
	10R1	*Streptomyces halstedii*	39.0%
	16R3B	*Streptomyces lavendulae*	62.3%
	17R1B/A	*Streptomyces lavendulae*	60.6%
	9R11	*Streptomyces violaceusniger*	41.0%
	19R2C	*Streptomyces* sp.	19.1%
	12R5/A	*Streptomyces* sp.	3.4%

[a]Identification was performed by fatty acid methyl ester (FAME) analysis and processed by MIDI Sherlock system. [b]Similarity Index: Positively identify, isolates identified with a SIM ≥ 0.3; tentatively identify, isolates identified with a SIM < 0.3 in Sherlock TSBA Library Version 4.0 (Misko and Germida 2002).

Endophytic Actinobacteria and Phytopathogenic Fungi *in Vitro* Interactions

An antagonism screening test was performed to evaluate if the endophytic strains were able to inhibit the phytopathogenic fungi growth. The results showed that *S. sclerotiorum*, *P. aphanidermatum*, *R. solani*, *Fusarium* sp. and *P. parasitica* were inhibited by 47.5, 55.0, 62.5, 77.5 and 90% of the isolates, respectively (Table 2). The results also demonstrated that five strains isolated from the leaves (18F3G/A, 14F1D/2, 19F2C/A, 14F1C/3 and 18F1D/A), three strains isolated from the roots (12R5/A, 10R1 and 19R2C) and one strain isolated from the stem (18C1C/B) was able to affect the mycelial growth of all the tested fungi. On the other hand, two strains isolated from the leaves (11F2A and 18F2A) were not able to inhibit any of the tested phytopathogens.

Although the strain 16R3B was not able to control *S. sclerotiorum* in the antagonism analysis, its secondary metabolites were able to inhibit the development of all the phytopathogenic fungi tested (data not shown). This strain along with the strain 14F1D/2 was chosen for further biological control analysis.

Table 2 - *In vitro* interaction between maize endophytic actinobacteria and phytopathogenic fungi.

Isolates	Phytopathogens				
	Pythium aphanidermatum	*Rhizoctonia solani*	*Sclerotinia sclerotiorum*	*Phytophthora parasitica*	*Fusarium* sp.
10R1, 18F3G/A, **14F1D/2**, 12R5/A, 18C1C/B, 19R2C, 14F1C/3, 19F2C/A, 18F1D/A	+	+	+	+	+
17R1B/A, 8F2	+	+	+	+	-
16R3B, 20C1A/B, 19F2C/1, 19F1C/2, 19C3B/D, P4F1, 9R1	+	+	-	+	+
15F3B, 18F2G	-	+	+	+	+
21C3A1, 20C1B, 21F2A, 19C3B/D'	-	+	-	+	+
20F3F	+	-	-	+	+
18F2C	-	-	+	+	+
16F3B, 14R3, 19C3B/C, 9R11	-	-	-	+	+
8R20, 19C3D/B, 14R2K	-	-	+	+	-
13C1B	-	+	-	+	-
13C18, 13C1B	+	-	-	+	-
18C2D, 18C1A/B	-	-	+	-	+
11F2A, 18F2A	-	-	-	-	-

Note: selected isolates are in bold.

Chitinolytic Analysis

Isolate 14F1D/2 displayed a 14.25 U chitinase activity whereas isolate 16R3B showed 10.3 U activity.

Biocontrol of *Pythium aphanidermatum* in Cucumber Seedling

Based on the *in vitro* screening tests, strains 14F1D/2 and 16R3B were selected for the biological control of *P. aphanidermatum* in cucumber seedlings, a host extremely susceptible to this pathogen. The fungicide control (phytopathogen + metalaxyl 3g a.i./L) and the negative control (treatment without the phytopathogen) did not show any damping off incidence (Table 3). Besides, the positive control (treatment with the phytopathogen) demonstrated 98% of damping off. The actinobacterium strain 16R3B was more effective biocontrol agent against this phytopathogen.

Table 3 – "Damping off" incidence in cumcumber seedlings.

Treatment	Incidence (%)
Positive Control	98.0 a
14F1D/2	64.0 b
16R3B	28.5 c
Fungicide Control	0.0 d
Negative Control	0.0 d

Note: Values with the same letter are not significantly different according to Tukey test ($p<0.05$). Fungicide Control = Metalaxyl (3g a.i./L).

The incidence of damping-off was reduced to 28.5% when this strain was used. The strain 14F1D was less effective in which only 36.0% of disease control was achieved.

DISCUSSION

The lack of genera diversity (34 out 40 isolates were classified as *Streptomyces* by FAME analysis) found could be result of the media used for the isolation - known to be selective for a few species (Matsumoto et al. 1998) and/or due to regional difference with regards to the climate and soil. Indeed, environmental factors pose a highly influence for isolation of actinobacteria (Xu et al. 1996; González et al. 2005). Besides, half of the isolates were recovered from maize leaves which indicated their ability to endophytically translocate inside the plant tissues. In a similar work, maize leaves were also the major reservoir for actinomycetes but representatives of three genera (*Microbispora*, *Streptomyces* and *Streptosporangium*) were recovered (Araújo et al., 2000). The divergence found between these communities of endophytic actinobacteria from the maize plants may be explained by the environmental differences between the two distant Brazilian regions were these studies were conducted: Northeast (tropical monsoon, Am; Araújo et al. 2000) and Southeast (humid subtropical, Cfa; this work). Nevertheless, these

discrepancies highlight the need to conduct more research to understand how these endophytic communities interact with maize plants in different edaphoclimatic conditions.

Many works have reported positive findings using *Streptomyces* species for controlling different plant pathogens (Berg et al. 2001; Bérdy 2005; Zucchi et al. 2008; Zucchi et al. 2010). Traditionally, *in vitro* tests are the first step for screening a new biocontrol candidate with antagonistic activity against the target phytopathogens (Kunoh 2002). Also, antagonism and antibiosis procedures are usually the most suitable methods for screening the antibiotics producing organisms for further commercial exploration of these by-products (Pliego et al. 2011). Almost all the endophytic isolates (95.1%) showed *in vitro* antagonistic effects against one, or more phytopathogenic fungi.

In an attempt to understand the mechanism involved in this *in vitro* interaction, the secondary metabolites produced by the endophytic actinobacteria were extracted and evaluated for their antibiosis effect. Strains 16R3B (*S. lavendulae*; SIM = 62.3%) and 14F1D/2 (*S. violaceusniger*; SIM = 68.3%) produced secondary metabolites, which were effective against all the phytopathogens tested and therefore, suggested that it might be one of the mechanisms used by these microorganisms to suppress the fungus development. Furthermore, both the strains produced chitinase, a well-known lytic enzyme used by the antagonist against the phytopathogenic fungi. The strains yielded almost the same amount of chitinase found for *Micromonospora carbonacea* and *Streptomyces viridodiasticus* (El-Tarabily et al. 2000), which were high chitinase producers (El-Tarabily and Sivasithamparam 2006).

The efficiency of strain 16R3B for the biological control of *P. aphanidermatum* in cucumber seedlings (71.5%) were similar to those found by Elad and Chet (1987). These authors evaluated the effect of 130 rhizobacterial strains isolated from several commercial crops against *Pythium* sp. and demonstrated that the incidence of damping-off decreased by up to 67%. This reduction was due to a competition between the microorganisms (biocontrol agent and phytopahtogen). In fact, other mechanisms besides the antibiosis and competition could be involved in controlling the phytopatogens by filamentous bacterial species, such as systemic resistance (Hasegawa et al. 2006; Conn et al. 2008). Actinomycetes producing lytic enzymes potentially hyperparasite the vegetative and/or reproductive structures of *Pythium* spp. (El-Tarabily et al. 1997). The selected strains used in this study were isolated from the leaf (14F1D/2) and root (16R3B) (Table 1). Although the highest number of endophytes was found in the leaves, the root isolate was the more effective against *P. aphanidermatum*. This could drive further efforts to better understand the interactions between the actinomycete endophytic community from the maize root and soilborne phytopathogens for selecting the biocontrol candidates.

The strain 14F1/D2, which had also shown promising results in laboratory conditions, failed when used in the greenhouse conditions. These discrepancies between *in vitro* and *in vivo* assays results have been discussed by many authors (Deacon and Berry 1993). One possible explanation for these differences could be that the biocontrol agent might be artificially favoured under the laboratory conditions (Weller 1988). This must be taken in to account before considering any isolate as a potential biocontrol agent. Despite of that the *in vitro* screening was effective in selecting a possible biocontrol candidate, isolate 16R3B. The results found under greenhouse conditions by this isolate highlighted its potential as a biocontrol agent to reduce the damping-off caused by *P. aphanidermatum* in this planting system.

CONCLUSION

The endophytic isolate 16R3B showed promising results *in vitro* and *in vivo* tests and therefore, this isolate could be considered as a potential candidate for further biological control programs of *P. aphanidermatum.*

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