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Antagonist actinomycetes metabolites against plant pathogens fungi of agricultural importance

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The study was conducted over the years 2012-2013 in order to evaluate the antagonistic effects of the metabolites of actinomycetes against phytopathogenic fungi of agricultural importance. To demonstrate the antagonist activity of antibiosis, bioassays were performed *in vitro* using the method of wells with cell-free filtrates and the double layer method with chloroform in order to kill living cells of actinomycetes and evaluate only the metabolites present in both methodologies. Three actinomycetes isolate previously selected by their antifungal activity APA2, AAH53 and APC70 were confronted with the fungus *Alternaria solani*, *Colletotrichum coccodes* y *Fusarium oxysporum.* All submitted actinomycetes antagonistic activity is in both methodologies, however greater antagonist activity was observed in trials with double-layer methodology, reaching up to 86% inhibition for *C. coccodes* and 72% for *A. solani*. Both have phytopathogenic fungi with actinomycete APC70. The analysis of three isolates of actinomycetes by comparing the sequences of 16S rRNA genes revealed that the isolates belong to the genus *Streptomyces.* Phylogenetic analysis revealed that strain is closely related APA2 *Streptomyces longisporoflavus*, isolation AAH53 with *Streptomyces mutabilis* and APC70 with *Streptomyces griseus*.

Key words: *Streptomyces*, antagonism, antibiosis bioassays, DNA.

INTRODUCTION

Actinomycetes are soil bacteria, of great importance in the rhizosphere; they can influence plant growth and protect roots against invasion by pathogens and in the formation and stabilization of composting and humus (Guo et al., 2015; Tarkka and Hamp, 2008). They are saprobiotic and endophytic bacteria that decompose the organic material specially biopolymers such as lignocellulose, starch and chitin in soil (Bentley et al., 2000; Passari et al., 2015). Some actinomycetes have similar morphological characteristics to fungi as mycelial growth culminating in sporulation; they are characterized by the production of bioactive extracellular components. Thousands of the secondary metabolites discovered are produced by actinomycetes, about 60% of new insecticides and herbicides reported in recent years are produced by *Streptomyces*. Actinomycetes produce various antibiotics with diverse chemical structures having antifungal, antitumor and immunosuppressive activities (Behal, 2000; Kitani et al., 2011).

Antagonist activity is usually related to the production of fungicides and extracellular hydrolytic enzymes, chitinases and β-1,3-glucanases which are considered as hydrolytic enzymes that cause lysis of the cell wall of fungi (El-Tarabily and Sivasithamparam, 2006; Prapagdee et al., 2008).

The genus *Streptomyces* produces a large number of secondary metabolites antagonists that are useful in the industry and the pharmaceutical (Seipke, 2015). Several groups of bioactive compounds such as macrolides, benzoquinones, aminoglycosides, polyenes and antibiotic nucleosides are examples of useful metabolites for agriculture produced by *Streptomyces* (Trejo et al., 1998; Ilic et al., 2007).

Kasugamycin is a bactericidal and fungicidal metabolite

discovered by Umezawa, in *Streptomyces kasugaensi* Hamada; this antibiotic acts as an inhibitor of protein biosynthesis in microorganisms but not in mammals, and his toxicological properties are excellent. It is currently used to control *Magnaporthe grisea* Hebert and bacterial diseases caused by *Pseudomonas, Burkholderia glumae* and fire blight of apple and pear caused by *Erwinia amylovora* in some crops (Yoshii et al., 2012). The Polyoxina B and D are metabolites that were isolated from *Streptomyces cacaoi* var. *asoensis* as a new class of natural fungicides, interfere with the synthesis of the cell wall of fungi specifically inhibit chitin synthetase. It has found applications against a number of pathogenic fungi in fruits, vegetables and ornamentals. The polyoxina D is produced by some companies to control sheath blight of rice caused by *Rhizoctonia solani* Kuhn. The antifungal metabolite mildiomycin isolated from a strain of *Streptoverticillium rimofaciens* Niida, is strongly active against *Oidium* sp. in various crops acting as an inhibitor of protein biosynthesis in fungi, has low toxicity in vertebrates so presents low impact on the environment (Doumbou et al., 2001; Li et al., 2012; Yoshii et al., 2012).

Efforts in the search for natural products for crop protection have a significant advance in the market and actinomycetes, especially those belonging to the genus *Streptomyces*; they appear to be good candidates to find new approaches to control plant diseases. The aim of this work focuses on the potential of actinomycetes as a source of agroactive compounds and tools in the biological control of plant diseases caused by fungi.

MATERIALS AND METHODS

Actinomycetes isolates

Three actinomycetes with previous antagonistic activity and antibiotic permanence over time were selected; APA2 (Isolated from air), AAH53 (Isolated from adult red…. ant *Atta* sp.) and APC70 (Isolated from sugarcane *Saccharum officinarum* L.). These isolates were grown on potato dextrose agar and kept in refrigerated storage.

Phytopathogenic fungi

Alternaria solani Sor, *Colletotrichum coccodes* Wallr and *Fusarium oxysporum* Schlech, phytopathogenic fungi, from the collection of the Department of Parasitology of the Universidad Autonoma Agraria Antonio Narro were used.

Preparation of fermentation extracts from actinomycetes

The three actinomycetes used in this work were propagated separately in Erlenmeyer flasks capacity 1L containing 200 ml of potato dextrose broth adjusted to pH 7.5 and incubated at 28°C to 150 rpm for 10 d. The fermentation broth was collected in sterile tubes and centrifuged at 15,000 rpm for 30 min in order to remove the cells. The cell-free supernatants were filtered aseptically through sterile polystyrene membranes with pore size of 0.45 µm (Prapagdee et al., 2008).

In vitro bioassays of antifungal activity of extracellular metabolites

Two methodologies to demonstrate the fungicidal capacity of the metabolic extracts, the method of wells and overlapping or double layer were used according to Williams et al. (1983) with some modifications to this study.

Wells method

Five (5) wells were done with sterile punchers in petri dishes containing PDA, each 200 µL of cell-free filtrate of each actinomycete separately; subsequently an explant 5 mm of each phytopathogenic fungus to evaluate was placed. The plates were incubated at 28°C for 5-10 d. Antibiosis was quantified by measuring with a digital Vernier, the diametral growth of the fungus to actinomycete strains compared to the development of the phytopathogenic fungus in the control (Prapagdee et al., 2008; Leon et al., 2011).

Overlap method or double layer

Different isolates of actinomycetes were individually planted in the four cardinal points of Petri dishes with PDA, incubated for 5 d at 28°C and then the colonies were exterminated by adding 1.5 mL of chloroform letting it act for 40 min. Deleted colonies were overlaid with 5 mL of PDA, when solidified a 5 mm explant of each phytopathogenic fungus was placed to be evaluated separately. The plates were incubated at 28°C for 7-15 d. Antibiosis was quantified by measuring with a digital Vernier the diametral growth of the fungus to actinomycete strains compared to control (Hayakawa et al., 2004; Selvakumar et al., 2010).

Identifying Actinomycetes

For identification of actinomycetes, a microscopic, macroscopic and molecular characterization was performed. For the macroscopic characterization were taken into account typical characteristics of the colonies of actinomycetes as texture, color, shape and size, while for microscopic, sterile coverslips were inserted with an

Table 1. Results of bioassays of antagonism from free-cells filtrates of selected actinomycetes against phytopathogenic fungi.

Actinomycete	Percentage of inhibition (%)					
	Alternaria solani		*Colletotrichum coccodes*		*Fusarium oxysporum*	
APA2	41.20	B ab	46.01	B a	30.63	A b
AAH53	47.59	AB a	50.00	A a	31.68	A b
APC70	54.13	A a	46.60	B b	21.33	B c

** Capital different letters in rows indicate significant difference p = 0.05; * Lowercase different letters in the columns indicate significant difference p = 0.05.

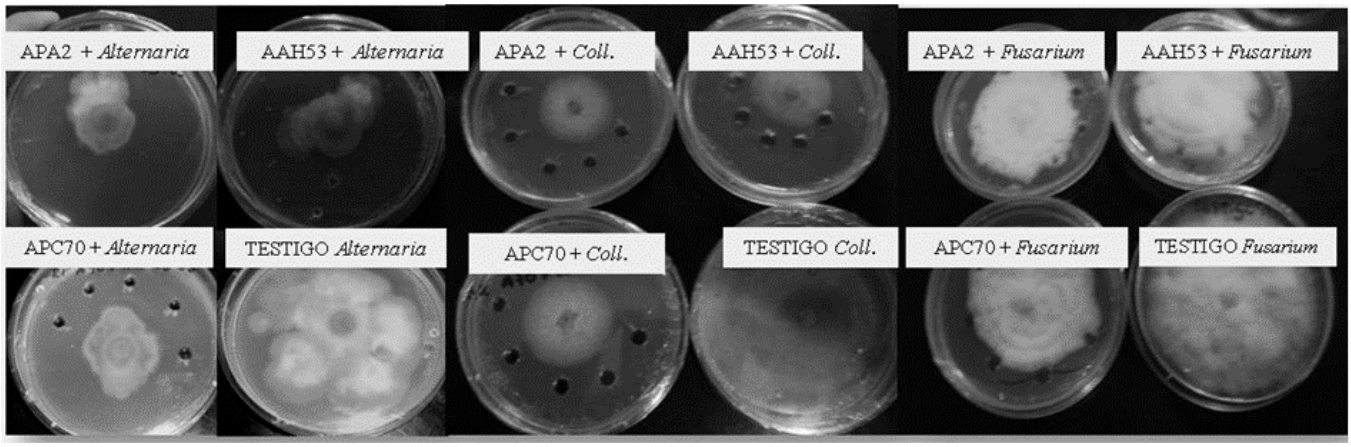

Figure 1. Bioassay of *in vitro* antibiosis by the methodology wells with cell-free extracts; (A) Antagonist effects of actinomycetes, APA2, ASH53 and APC70 compared with the control fungus *Alternaria solani*; (B) Antagonist effects of actinomycetes APA2 , ASH53 and APC70 compared with the control fungus *Colletotrichum coccodes*; (C) Antagonist effects of actinomycetes APA2, ASH53 and APC70 compared with the control fungus *Fusarium oxysporum.*

inclination of 45° to the agar surface. The plates were incubated at 28°C for 8-15 d, and then coverslips were taken and placed on a slide with crystal violet for microscopic observation. They were taken into account in addition to the Gram stain, aerial and vegetative mycelium, fragmentation of the mycelium, a group of spores, presence of spirals, terminal spores, in pairs or groups in the mycelium (Bergey, 2000; Franco-Correa et al., 2010).

For molecular identification, a DNAr 16S amplification was performed by PCR using as template the genomic DNA extracted directly from the actinomycete cells. The primers used for the PCR reaction were 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1510R (5'-GGCTACCTTGTTACGA-3'). Amplification was performed in a final volume of 50 µL containing 10 µL of Buffer 10X, 2 µL of nucleotide 10 Mm, 10 µL of $MgCl_2$ 25 mM, 2 µL of each primer (9F and 1510R), 0.8 µL of Taq DNA Polymerase, 5 µL of DNA sample (25 ng total DNA) and distilled water to complete the 50 µL. The conditions for the thermal cycler included: DNA denaturation at 96°C for 2 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing 55°C for 1 min and extension 72°C for 1 min, and an end cycle of 72°C for 5 min, was maintained at 4°C (Retnowati, 2010; Srivibool et al., 2010). Waiting a product of 1500 bp, the PCR products were sequenced and subjected to BLAST analysis and their sequences compared to the GenBank database.

RESULTS AND DISCUSSION

Antibiosis bioassays

Wells method with cell-free filtered

Results of antagonism of actinomycetes isolates are shown in Table 1, which shows that the inhibitory activity ranges from 21.33 to 54.13% depending on the phytopathogenic as in the isolate APC70 against *A. solani* which had the highest percentage of inhibition, followed by actinomycete AAH53 against *C. coccodes* fungus with a percentage of 50% inhibition (Figure 1), similar to that reported by Prapagdee et al. (2008), with *Streptomyces hygroscopicus* against *Colletotrichum*

Table 2. Results of bioassays for double layer antibiosis of selected actinomycetes against phytopathogenic fungi.

Actinomycete	Percentage of inhibition (%)					
	Alternaria solani		***Fusarium oxysporum***		***Colletotrichum coccodes***	
APA2	67.17	AB b	60.81	A c	81.18	B a
ASH53	59.16	B a	48.95	B b	65.09	C a
APC70	72.02	AB b	59.99	A c	86.18	A a

** Capital different letters in rows indicate significant difference p = 0.05; * Lowercase different letters in the columns indicate significant difference p = 0.05.

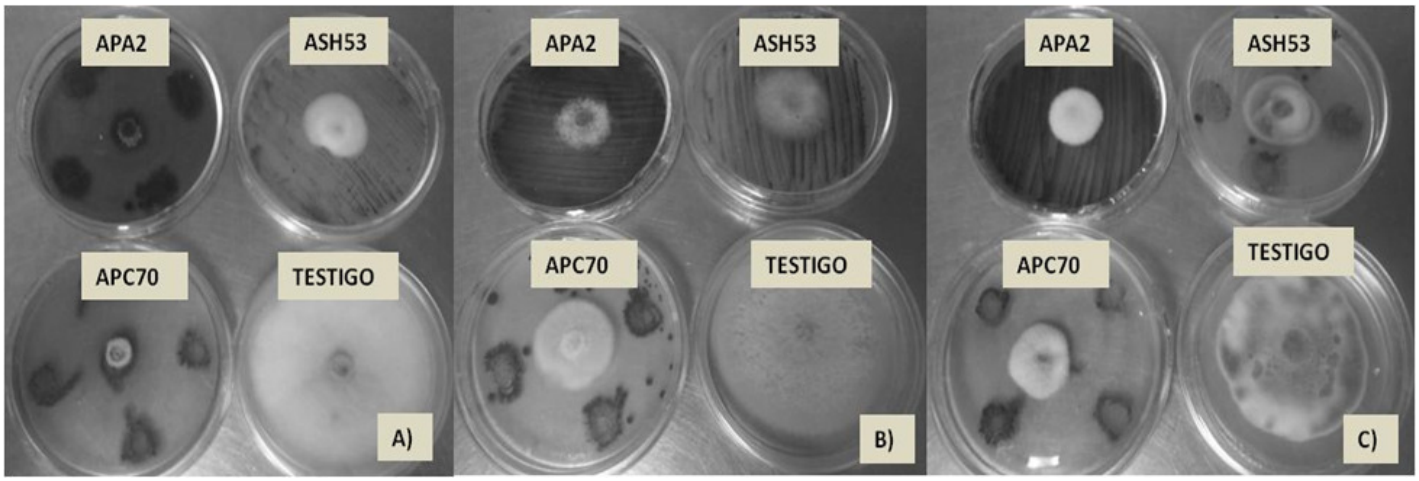

Figure 2. Bioassay of in vitro antibiosis by double-layer methodology. (A) Antagonist effects of actinomycetes APA2, ASH53 and APC70 compared with the control fungus *Colletotrichum coccodes;* (B) Antagonist effects of actinomycetes APA2, ASH53 and APC70 compared with the control fungus *Fusarium oxysporum;* (C) Antagonist effects of actinomycetes APA2, ASH53 and APC70 compared with the control fungus *Alternaria solani.*

gloeosporioides, in bioassays with cell-free filtrates, as Getha and Vikineswary (2002) who reported to *Streptomyces violaceusniger* G10, with antagonism against *F. oxysporum* f. sp. *cubense,* like the isolates APA2 and AAH53 due to the production of extracellular metabolites antifungals. Rodriguez et al. (2014) showed that *Streptomyces* sp. presents an antagonistic effect against different phytopathogenic fungi, with percentages of inhibition of 60 to 100%.

Actinomycetes produce metabolites that degrade chitin, which is a component of the cell wall of fungi, like chitinase, extracellular enzyme that break down the cell wall of fungi (Hoang et al., 2011). Mahadevan and Carwford (1997) determined the chitinase production by *Streptomyces lydicus*, showing that chitinase and other metabolites play an important role in biocontrol of plant pathogenic fungi.

Overlap method or double-layer

All isolates of *Streptomyces* sp. showed significant antagonistic effects on plant pathogenic fungi tested, showing statistical differences depending on fungus and the species of actinomycetes, with highest percentages presented by APC70 and APA2 isolates against *Colletotrichum* sp. with 81.18 and 86.18% respectively; *Alternaria* sp. also was inhibited successfully with 72.02% inhibition, and to a lesser extent for *F. oxysporum* with 60% inhibition (Table 2; Figure 2).

Sporulation is directly related to the production of secondary metabolites and the antibiotic is within spores (Procopio et al., 2012). Hence there are factors which depend on the levels of antagonism produced by actinomycetes that can affect sporulation of these and therefore the production of secondary metabolites, culture medium, incubation time, temperature, pH, etc. Tanaka and Omura (1990) published a study showing that strains of *S. griseus* and *S. hygroscopicus* are higher than other actinomycetes in their ability to produce a large number and variety of bioactive metabolites, in this way it can be explained why the percentage of antagonism of this research has differences depending on isolate of actinomycetes evaluated and, therefore of the species. According to studies by Chamberlain and Crawford (1999) and Crawford et al. (1999).

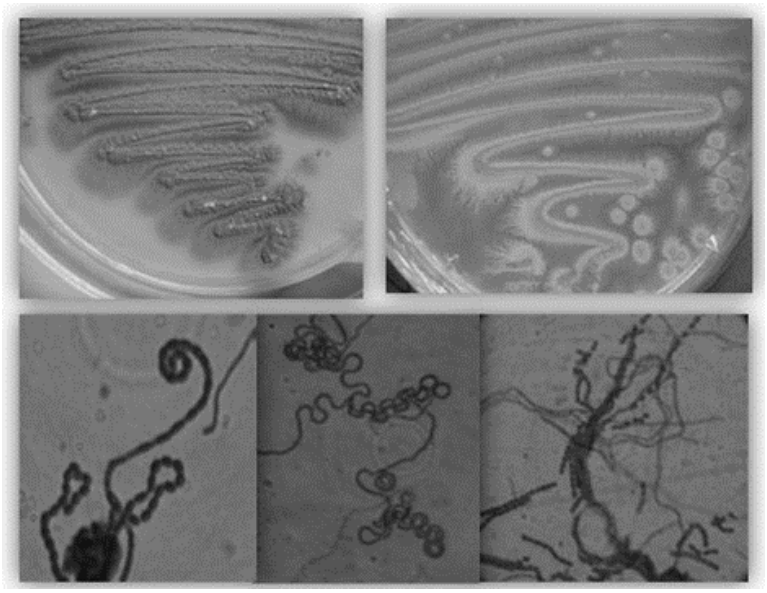

Figure 3. (A) Macroscopic morphology of isolate ASH53, dry colony, dusty, irregular with grayish pigment; (B) Macroscopic morphology of isolate APC70, dry dusty colony, white; (C) Microscopic morphology, isolate ASH53, vegetative and aerial mycelium, not fragmented, forming spiral spore chains; (D) Microscopic morphology, isolate APA2, tortuous mycelium; (E) Microscopic morphology, isolate APC70, vegetative and aerial mycelium, forming long chains of spores (Observed in brightfield 12,000 X).

The production of antibiotics by *S. hygroscopicus* can inhibit a wide range of fungal pathogens such as *Rhizoctonia solani, Pythium ultimum, F. oxysporum,* and *Sclerotinia homeocarpa*.

Hayakawa et al. (2004) performed bioassays *in vitro* for antimicrobial activity using the bi-layer methodology leading to an inhibition of all pathogens evaluated, which were bacteria, yeasts and filamentous fungi as *Aspergillus niger* and *Penicillium chrisogenum.* Similarly Selvakumar et al. (2010) used this methodology, but to control *Aeromonas hydrophila* a fish pathogen that causes human poisonings presenting results of considerable inhibition.

Macroscopic and microscopic identification

The colonies that had irregular edges, showed a hard and compact consistency and were adhered to the surface of the medium; they also presented a powdery appearance due to the formation of long chains of spores highly hydrophobic (Figure 3). Isolate APA2 showed a brown pigment diffused in the medium and AAH53 presented grayish colonies, other isolates showed white color colonies. The variety of colors is due to the ability of these organisms to produce melanoid pigments and to the presence of different species (Sylvia, 2005).

Microscopic features observed were vegetative and aerial mycelium; tortuous mycelium unfragmented forming spiral spore chains and long chains of spores (Figure 3). All isolates tested were Gram +. Macroscopic and microscopic characteristics were compared with those described in the Manual of bacteriological determination of Bergey (2000). The results show that the isolates have similarity to the morphological characteristics of the genus *Streptomyces.*

Molecular identification

Amplification of DNAr 16S of the three isolates of actinomycetes generated a fragment of approximately 1500 bp which was consistent with that reported by Retnowati (2010) and Sirvibool et al. (2010) as shown in Figure 4. The results were subjected to BLAST analysis using the NCBI database to identify to genus and species. Isolates had different nucleotide sequences for the DNAr 16S gene, indicating that they were different species. Isolate APA2 is closely related to *Streptomyces longisporoflavus,* with 94% similarity; isolate AAH53 to *Streptomyces mutabilis* with a 99% similarity and APC70 with 99% compared to the sequence of *Streptomyces griseus.*

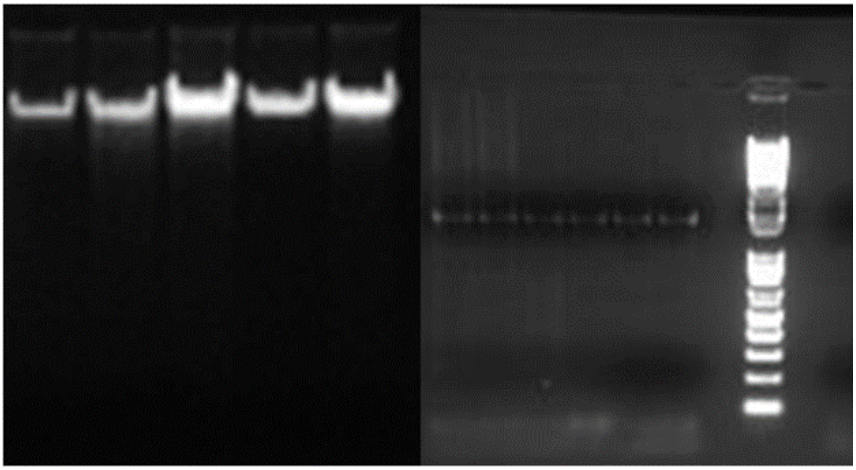

Figure 4. (A) Gel of DNA quality of selected actinomycetes; (B) Agarose gel of PCR products obtained from amplification of DNA of actinomycetes samples using primers 9F and 1510R. Line 1 and 2: APA2, Line 3 and 4: ASH53, Line 5 and 6: APC70, Line 7: Negative Control Line 8: molecular marker 12,000 bp.

Conclusion

Actinomycetes, especially those belonging to the genus *S. longisporoflavus*, *S. mutabilis* and *S. griseus* produce bioactive secondary metabolites against pathogenic fungi *A. solani, F. oxysporum* and *C. coccodes*.

The method of bilayer to assess the ability of actinomycetes facilitates determining the antagonist activity of these microorganisms in their own culture plate where the antagonistic determination is made, because it is done with solid medium which promotes the production and distribution of secondary metabolites.

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