International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 12 (5), pp. 001-006, May, 2018. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

In vivo and *in vitro* antagonism of *Streptomyces* sp. RO3 against *Penicillium digitatum* and *Geotrichum candidum*

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Accepted 22 March, 2018

***Streptomyces* strains may control postharvest lemon fruit diseases caused by *Penicillium digitatum* and *Geotrichum candidum*. Four *Streptomyces* strains (RO3, MC3, Alem and CE1) were tested and RO3 was the strain with the highest antagonistic activity. *Streptomyces* RO3 showed a maximum biomass value (2.15 g/L) after 72 h incubation in yeast extract, malt extract, glucose (YMG) medium; in starch casein medium the highest value was 0.477 g/L at 120 h incubation. The metabolites produced in YMG medium showed the maximum inhibition against *G. candidum* and *P. digitatum* (19.64 and 62.12% respectively). The inhibitory activity of metabolites decreased after autoclaving. RO3 metabolites have molecular mass higher than 2000 and they have fungicidal action mode against *P. digitatum*. *In vivo* assays, RO3 metabolites with wax, totally controlled lemon diseases (mainly sour rot and green mold). This is the first study on *Streptomyces* to control postharvest lemon diseases.**

Key words: *Streptomyces*, *Penicillium digitatum*, *Geotrichum candidum*, metabolites, lemon diseases.

INTRODUCTION

Injuries sustained by citrus fruit during harvest, allow the entry of wound pathogens like *Penicillium digitatum* and *Geotrichum candidum*, causal agents of green mold and sour rot, respectively (Plaza et al., 2004). Traditionally, these diseases are controlled by the application of fungicides during fruit processing. However, several chemicals (like: 2-4 thiazalil benzimidazole, imidazole) are no longer used for postharvest treatment or have been removed from the market altogether, due to possible toxicological risks (Lingk, 1991). Alternative control methods are needed because of negative public perceptions about the use of pesticides, development of fungicide resistant fungal pathogens and high development costs for new chemicals. In recent years, biological control of postharvest fruit diseases has become an important field of research. A number of yeasts and bacteria (*Bacillus subtilis*, *Agobacterium radiobacter, Candida guillermondii, Kloeckera aiculata, Cryptococcus laurentii, Rhodotorula glutinis*) have been reported to inhibit fruit postharvest decay effectively (Janisiewicz and Korsten, 2002). Modes of action involved may be secretions of antibiotics (Trejo-Estrada et al., 1998a), competition for space and nutrients (Zang et al., 2005) and production of cell wall lytic enzymes (Saligkarias et al., 2002) and induction of host resistance (Wilson et al., 1994). *Streptomyces* is the best known and most studied genus of the Actinomycete family. *Streptomyces* strains present a series of characteristics that would make it a very good biocontrol agent against phytopathogens (Grabley et al., 1999; Lee et al., 2002). *Streptomyces* strains showed a protective effect on tomatoes (Minuto et al., 2006; Sabaratnam et al., 2002), wheat (Coombs, 2003), potatoes (Jobin et al., 2005; Shahrokhis et al., 2005) and lettuce (El-Tarabily et al., 2000). No data was found on *Streptomyces* to control pre or postharvest citrus diseases. The purpose of this research was the potential of *Streptomyces* strains to control postharvest lemon fruit diseases caused by *P. digitatum* and *G. candidum*. In addition, we studied

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possible modes of action involved in postharvest pathogen control.

MATERIALS AND METHODS

Bacterial and fungal strains

Streptomyces strains RO3, MC3, Alem and CE1 provided by Instituto de Microbiologia of UNT strain collection (Tucuman, Argentina) maintained in Sabouraud at 4°C were used. A cell suspension of *Streptomyces* strains was prepared in saline solution and ajusted to 1.5 10^8 cells/ml corresponding to 0.5 of Mc Farland scale.

P. digitatum and *G. candidum* were obtained from the Instituto de Biotecnología of UNT strain collection (Tucuman, Argentina) and kept in PDA at 4°C. Spores were harvested by flooding the surface of 10 days-old cultures with distilled water; the inoculum concentration used was 10^6 spores/ml. This concentration is commonly used in citrus postharvest experiments (Eckert and Brown, 1986).

In vitro assays of antagonism in liquid medium

Fifty milliliters of starch casein medium (g/L: starch, 10; casein,1; K_2HPO_4, 0.5), pH 7.2 were inoculated with *Streptomyces* strains (1.5 10^8 cells/ml) and incubated for 5 days (250 rpm, 30°C). Two assays were performed to identify the best metabolite producer strain. (A) Five milliliters of *Streptomyces* culture of each strain and (B) Five mililiters of cell free culture broth obtained by centrifugation at 200 rpm, 20 min were used for antagonism tests.

In Erlenmeyer flasks, 40 ml of potato dextrose broth and 5 ml of antagonist (A or B) were inoculated with 5 ml of *P. digitatum* and *G. candidum* (10^6 spores/ml) and incubated in shaker at 250 rpm, 30°C for 5 days. The biomass was separated with Whatman Nº 5 filter paper and washed repeatedly with distilled water. It was manteined at 102°C, 24 h to determine dry weight. The results were expressed as inhibition percentages.

Growth kinetics and metabolites production for *Streptomyces* RO3

Kinetics studies were carried out with starch casein and YMG media (g/l : yeast extract, 4; malt extract,10; glucose, 4; K_2HPO_4 , 2; casaminoacids, 0.1%; pH 7.2) to optimize *Streptomyces* RO3 growth and metabolites production. 50 ml of YMG and starch casein media were inoculated with 5 ml of a cell suspension (1.5 10^8cel/ml) of *Streptomyces* RO3, incubated for 8 days at 30°C, samples were taken every 24 h. Biomass was separated by filtration and then was dried. The supernatant was used to carry out antagonism assays in solid medium and *in vivo.*

In vitro antagonism in solid medium

A dual culture was carried out following the method described by Aghighi et al. (2004). *P. digitatum* and *G. candidum* solid culture agar plugs (6 mm), were placed in the center of Petri dish with APG medium, and at 30 mm distance from it, three *Streptomyces* RO3 agar plugs of 4 - 6 days solid culture (Sabouraud medium) were placed. In the second assay *Streptomyces* strain was incubated during 3 days at 37°C and then the agar plugs with phytopathogenic fungi (*P. digitatum* and *G. candidum*) were placed in the middle of Petri dish. They were incubated at 30°C for two weeks. Control assays included fungal mycelial plugs in center of non-Actinomycete inoculated PDA plates (El-Tarabily et al., 2000; Lee et al., 2002). Antifungal activity was defined as growth inhibition against pathogenic fungi and was calculated by subtracting the radius distance (mm) of fungal growth obtained in the control and antagonist plates. Reference values to evaluate inhibition were: slight (5 - 9 mm), moderate (10 - 19 mm) and strong (> 20 mm).

In vivo antagonism assays

In vivo assays were carried out with RO3 strain to study its inhibitory activity against *P. digitatum* and *G. candidum*. Fresh EUREKA lemons of uniform size and maturity, without wounds, were used in this study. They were harvested in July, August and September 2007 from Tucuman, Argentina. Four batches of 10 lemons each were used: (1) Lemons without treatment (control disease); (2) Lemons treated with solution of sodium bicarbonate (200 ppm) and chemical fungicide (imazalil 4000 ppm) (control without disease); (3) Lemons treated with 50 ml of sodium bicarbonate (200 ppm), plus 50 ml of metabolites obtained after centrifugation (200 rpm) of *Streptomyces* culture; (4) Lemons treated with 50 ml of sodium bicarbonate (200 ppm) with 50 ml of *Streptomyces* culture; (5) Lemons treated as in 3 and 4, but with the addition of 18% wax-water emulsion. The fruits were sprayed, allowed to dry, placed in boxes with moistened paper towels to maintain 85% humidity and covered with plastic. They were kept for 3 weeks at 4°C and 1 week at room temperature to reproduce the storage conditions until reaching their final destination. The results were expressed as percentage of diseased lemons. The results reported are the average of triplicate determinations.

Heat stability assay

To determine RO3 metabolites heat stability, they were filtered or sterilized at 120°C, 15 min. Their inhibiting activity was evaluated only against *P. digitatum.*

Metabolite molecular mass estimation

A benzoylated dialysis tube (SIGMA) capable of separating molecular mass compounds between 1200 and 2000 M.W. was used. Five millilitres of metabolites were dialyzed at 4°C, 6 h in phosphate buffer 0.2 M pH 7. Recovered metabolites from the dialysis tube were sterilized by filtration and assayed for *in vitro* antagonism against *P. digitatum*.

Determination of metabolite mode of action

It was carried out according the method described by Lacasa et al. (2001) and Pastos (2005). One milliliter spore suspension of *P. digitatum* (10^3 spores/ml) in dextrose potato broth and 1 ml of RO3 metabolites were incubated at 30°C, 5 days. A control was prepared with 1 ml of spore suspension and 1 ml of distilled water. Samples were centrifuged, washed and spread in PDA dishes. The PDA dishes were incubated at 30°C, 5 days, to determine if metabolite's effect was fungicide or fungistatic.

RESULTS

The *Streptomyces* culture from each strain (MC3, RO3, CE1 and Alem) and their metabolites were tested against

Table 1. Antifungal activity of *Streptomyces* RO3 metabolites and *Streptomyces* RO3 culture against *P. digitatum* and *G. candidum*.

	Inhibition (%)	
	RO3 metabolites	***Streptomyces* RO3 culture**
P. digitatum	28.54	63.16
G. candidum	19.71	60.79

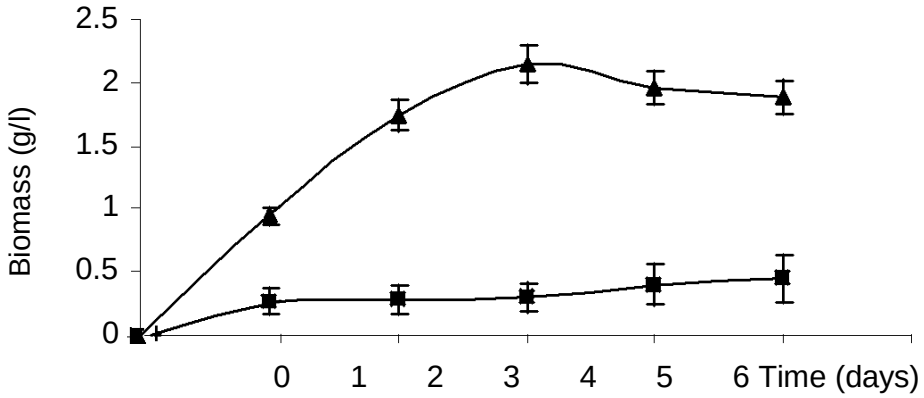

Figure 1. Growth kinetics of *Streptomyces* RO3 strain in Starch Casein ( ) and YMG ( ) media pH 7.2 at 30ºC during 8 days.

P. digitatum. The strain with the highest antagonistic activity was RO3 (data not shown).This strain was chosen for further studies.

RO3 *in vitro* antagonism against several fungal strains

The *Streptomyces* RO3 culture and its metabolites against *P. digitatum* and *G. candidum* were studied and both could inhibit fungal growth. Table 1 shows that the inhibitory effect was higher in the confrontations carried out with *Streptomyces* RO3 culture.

Streptomyces RO3 growth and metabolites production in YMG and starch casein media.

Growth kinetics for RO3 in YMG medium showed a maximum biomass value of 2.15 g/l after 72 h incubation, while in starch casein medium the highest value was 0.477 g/l after 120 h incubation (Figure 1). The metabolites obtained from 1 to 8 days fermentations in starch casein and YMG media were assayed against *P. digitatum* and *G. candidum* in liquid media antagonism tests. The metabolites produced in YMG after 24 to 48 h incubation showed the maximum inhibition against *G. candidum* and *P. digitatum* (19.64 and 64.12% respectively) while those produced in starch casein presented the highest inhibition levels after 5 days fermentation (17.82 and 18.87%, respectively) as seen in Table 2. All metabolites from more than 5 days fermentation kept inhibitory activity against both phytopathogens, but their inhibition percentages were lower than the maximum values obtained in previous days.

Antagonism assays in solid medium

In both antagonism assays, strain RO3 inhibited *P. digitatum* and *G. candidum* growth. At the dual culture assay, the inhibition was moderate to slight, according to the reference scale. In the previous antagonist incubation assay, the growth inhibition levels were up to 39% for *P. digitatum* and 29% for *G. candidum* (Figure 2). Colour observed in plate B was due to the characteristic diffusible pigment production of *Streptomyces* strain.

In vivo antagonism assays

Lemons without treatment showed disease incidence percentages of 70 to 90%, while lemons with chemical fungicide treatment showed no signs of disease. When biological treatment was applied, the results were as

Table 2. Inhibition (%) produced by of Streptomyces RO3 metabolites from YMG and starch casein media against *P. digitatum* and *G. candidum*.

Days		Inhibition (%)							
		1	2	3	4	5	6	7	8
P. digitatum	Metabolites from YMG	59.64	64.12	48.51	25.77	22.3	12.77	12.21	10.33
	Metabolites from starch casein	1.52	1.77	4.02	10.26	18.87	9.08	7.71	13.43
G. candidum	Metabolites from YMG	19.64	12.52	17.09	14.28	17.54	16.89	18.11	17.34
	Metabolites from starch casein	0	0	6.5	12.21	17.82	14.33	13.85	16.59

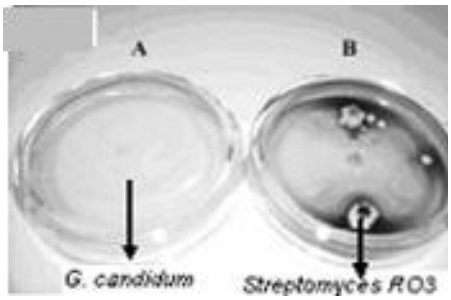

Figure 2. Antagonism assay in solid medium between G*. candidum* and *Streptomyces* RO3 strain (Plate B). Plate A: control.

follows: (1) *Streptomyces* culture without wax reduced disease incidence 20%; with wax, the disease was reduced 10%. (2) Metabolites without wax produced disease incidence levels of 10%; with wax disease was totally controlled (Figure 3).

Heat stability

Streptomyces metabolites were affected by heat treatment. Their inhibitory activity decreased sharply after autoclaving. *P. digitatum* growth inhibition percentages for autoclaving and filtration treatments were 8.32 and 61.7% respectively (Figure 4).

Molecular mass assessment for RO3 metabolites

P. digitatum growth inhibition produced by RO3 metabolites showed identical levels as observed without dialysis and after this treatment. We assumed that benzoylated dialysis tube retained RO3 metabolites and they have a molecular mass higher than 2000.

Metabolites mode of action

P. digitatum was unable to grow in PDA medium after their spores were exposed to RO3 metabolites at 30°C, 5 days. Based on that result we assumed that RO3 metabolites presents fungicidal mode of action.

DISCUSSION

In vitro assays *Streptomyces* RO3 metabolites presents higher inhibitory effect than *Streptomyces* culture. This effect could be attributed to competition for space, nutrients, etc. The YMG medium was better than starch casein medium for *Streptomyces* growth and its metabolites production. These results partially coincide with those obtained by Sabaratnam et al. (2002); the authors also observed that maximum biomass production occurred after 3 days fermentation in identical conditions. The highest *Streptomyces* RO3 biomass was 2.15 g/l in YMG medium after 72 h incubation, but grew poorly in starch casein medium, which, Sabaratnam et al. (2002) suggested as better than other media.

The metabolites produced in YMG showed higher inhibitory activity against *P. digitatum* and *G. candidum*. The different chemical composition of YMG and starch casein media used for metabolites production could have a direct influence on their antimicrobial activity spectrum. Trejo-Estrada et al. (1998b) indicated that some nutrients like glucose, ammonia, phosphate and trace metals are important in the regulation of *Actinomycetes* secondary metabolism. In the case of dual culture technique, only moderate to slight inhibitions were observed. Aghighi et al. (2004) found that *Streptomyces* metabolites assayed in solid medium tests presented a slight inhibition against *Fusarium solani*, but it was moderate to strong against *Phytophtora megasperma*. With the previous antagonist incubation assay, the results were better than the obtained with dual culture. Trejo-Estrada et al. (1998) demostrated that *Streptomyces violaceusniger* YCED9 showed 50% inhibition with this same technique against different phytopathogens (*Rhizoctonia solani, Gaeumannoyces graminis, Pythium ultimun* and *S. homeocapa*).

When treatments were carried out *in vivo*, citrus damage caused by *P. digitatum* and *G. candidum* was considerably reduced and wax addition contributed to

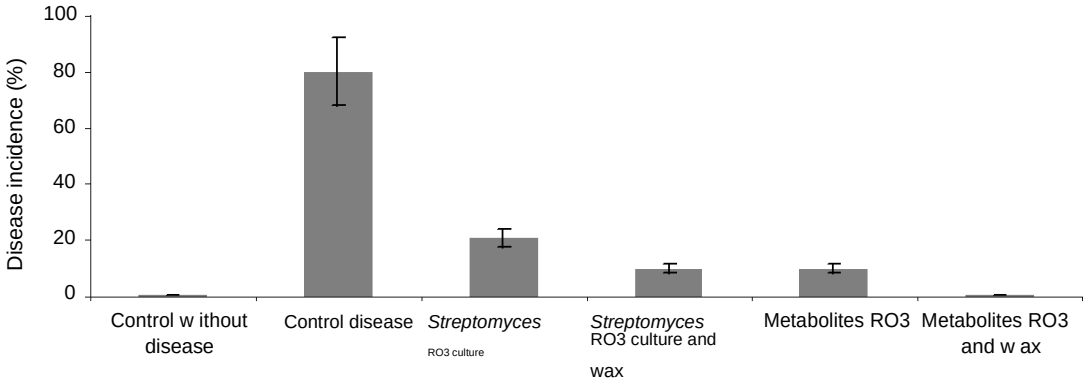

Figure 3. *In vivo* assays of RO3 metabolites and *Streptomyces* RO3 culture treated and no treated with wax against *P. digitatum* and *G. candidum.*

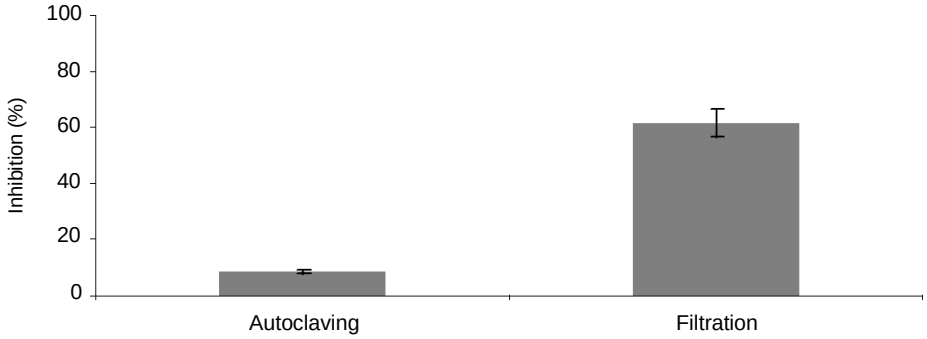

Figure 4. Antagonism assay from *Streptomyces* RO3 metabolites sterilized by autoclaving and filtration, against *P. digitatum*.

fruit preservation, due to avoid lemon dehydratation. In this case, better results were achieved with *Streptomyces* RO3 metabolites, in faced to *in vitro* assays. Plaza et al. (2004) pointed out that it is not possible to extrapolate all *in vitro* results to natural systems since *in vivo* there are other factors, besides those in the environment as the nature of its components, interaction with other microorganisms, etc.

The biological treatment applied may be compared to the chemical one used in packing houses. RO3 metabolites were inhibited by heat; this effect could be due to their chemical nature and/or to a possible inhibition of lytic enzymes presents in fermentation broth. Mahadevan et al. (1997) and Trejo Estrada et al. (1998) founded that, although antibiosis is one of the main mechanisms found in *Streptomyces*, the production of lytic enzymes (chitinases, 1-3 glucanases) capable of acting on the fungus cell wall by altering growth and viability is also common among them. *Streptomyces* RO3 metabolites have molecular mass bigger than 2000 and their mode of action is fungicidal. Several authors report *Streptomyces* ability to produce fungicidal substances like Streptomycin, Kasugamycin, Polyoxin and Validamycin that have different action modes and are the active ingredients of many biological plaguicides (Duran, 2004).

The purpose of this work was to prove *Streptomyces* RO3 potential to reduce or eliminate some of the main citrus postharvest diseases (green mold and sour rot). Further studies are needed in order to determinate the nature of *Streptomyces* RO3 metabolites and their mechanism of action; this biological agent could be an alternative to the synthetic fungicides used in packing house.

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