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Full Length Research Paper

Biological control of citrus green mould, *Penicillium digitatum*, by antifungal activities of Streptomyces isolates from agricultural soils

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Streptomyces strains can control postharvest citrus fruit diseases caused by *Penicillium digitatum*. One hundred and ten (110) *Streptomyces* strains were isolated from agricultural soils and screened against *P. digitatum*. The antagonistic activities of the isolates were determined through dual culture technique. In the 110 isolates, only strain 328 showed strong antifungal inhibitory effects. *Streptomyces* strain 328 showed a maximum biomass value (1.85 g/L) after 72 h incubation in yeast extract, malt extract, glucose (YMG) medium; in starch casein medium, the highest value was 0.68 g/L after 96 h incubation. The metabolites produced in starch casein medium showed the maximum inhibition zone against *P. digitatum* (28 mm diameter). *Streptomyces* strain 328 metabolites have molecular mass higher than 2000 and they have fungistatic mode of action against *P. digitatum*. In *in vivo* assays, the selected isolate has significantly reduced severity of green mold on crops which have been stored under 24°C for 28 days as compared to the controls. The inhibition effect of about 95% was shown by strain 328.

Key words: *Penicillium digitatum*, *Streptomyces* spp., Actinomycetes, biocontrol.

INTRODUCTION

Postharvest losses of fresh fruits and vegetables may reach very high values depending on species, harvest methods, length of storage and marketing conditions, Postharvest diseases often account for a major part of losses (Brown and Chambers, 1996; Bull et al., 1997; Pailly et al., 2004) and their control requires use of a large amount of fungicides (Wilson et al., 1993; Holmes and Eckert, 1999). Biological control of postharvest diseases by antagonistic microorganisms seems to be a promising alternative to fungicides (Qing and Shiping, 2000; Fan and Tian, 2001; Long et al., 2005; Zhang et al., 2005). Understanding the methods of action of antagonisms is essential to allow the use of antagonists under partial conditions and to enhance their biological control while protecting the human health and the environment. Several modes of action have been documented for the antagonistic activity of biological control agents: they act by multiplying on the fruit surface or within wounds on the fruit, then by competing for space and nutrient at an infection court on the

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commodity, by antibiosis, by restricting the action of hydrolytic enzymes produced by the pathogen, by producing enzyme to degrade pathogen cell walls, and/or by direct parasitism of the pathogen (Jijakli and Lepoivre, 1998; Bar-Shimon et al., 2004; Long et al., 2005). Green mould, caused by *Penicillium digitatum* Sacc., is among the most economically important postharvest diseases of citrus worldwide. It has been estimated that losses of citrus fruit due to green mould infection is 30-50% in China (Boxun et al., 2002), the world's largest producer of citrus fruit. One major research focus in this area has been the selection and development of effective and environment-friendly agents for biological control of this disease. The purpose of these experiments was to evaluate the commercial potential of Streptomycetes for the control of postharvest decay of citrus fruit. At present, we have targeted our efforts toward the control of green mould.

Streptomyces spp. are one of the most attractive sources of biologically active substances such as vitamins, alkaloids, plant growth factors, enzymes and enzyme inhibitors (Omura, 1986; Shahidi, 2003). Soil Streptomycetes are one of the major contributors to the biological buffering of soils and have roles in decomposition of organic matter conductive to crop production (Gottlieb, 1973; Kieser et al., 2000). For this research, 110 isolates of *Streptomycetes* were isolated from agricultural soils of Kerman of Iran and screened against *P. digitatum in vitro* and *in vivo*. The objective of this study is to isolate *Streptomyces* strains having antagonistic properties with the aim that they can serve as gene donors in developing resistant transgenic plants and used for soil amendments as biofertilizer or biofungicide in biological control of the tested pathogen. From all tested isolates of *Streptomycetes*, 11 *Streptomyces* strains showed high *in vitro* anti green-mold activity.

MATERIALS AND METHODS

Culture media

P. digitatum was obtained from Iran Plant Protection Research Institute (IPPI). The pathogen was cultured on potato dextrose agar (PDA). 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). Casein glycerol agar (CGA) was prepared from basic ingredients as described by Küster and Williams (1964) and used as *Streptomycetes* culture.

Soil sampling and isolation of Streptomycetes

Samples of soil were collected from grassland, orchards and vegetable fields in different parts of Kerman, Iran (Kerman is one of the major region that produces varied agricultural crop, particularly citrus). Several samples were selected using an open-end soil borer (20 cm in depth, 2.5 cm in diameter) as described by Lee and Hwang (2002). Soil samples were taken aseptically from a depth of 10-20 cm below the soil surface. Samples were air-dried at room temperature for 7 to 10 days and then passed through a 0.8 mm mesh sieve and were preserved in polyethylene bags at room temperature. Ten gram samples were mixed with sterile distilled water (100 ml) and the mixtures were shaken vigorously for one hour and then allowed to settle. One milliliter of soil suspensions (diluted 10^{-1}) were transferred to 9 ml of sterile distilled water and subsequently diluted to 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Inoculum consisted of adding aliquots of 10^{-3} to 10^{-6} soil dilutions to autoclaved CGA (1, 25 ml^{-1}CGA) at 50°C and then poured into plates. Three independent replicates were considered for each dilution. Plates were incubated at 30°C for up to 20 days. The *Streptomyces* colonies were isolated and incubated at 28°C for one week and stored in a refrigerator as pure cultures (growing a single clone on medium is indicative of pure culture) and at the end 110 *Streptomyces* isolates were screened.

In vitro studies

To estimate the antifungal activity of the *Streptomyces* isolates against pathogen, bioassays were done using agar disk method as described by Shahidi Bonjar (2003) and Aghighi et al. (2004) and the ratings used were modified by Lee and Hwang (2002) and El-Tarabily (2000). 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 to 9 mm), moderate (10 - 19 mm) and strong (> 20 mm).

Monitoring activity

Growth kinetics and metabolites production for Streptomyces strain 328

Kinetics studies were carried out with starch casein and YMG media (g/l : yeast extract, 4; malt extract,10; glucose, 4; K2HPO4 , 2; casaminoacids, 0.1%; pH 7) to optimize *Streptomyces* strain 328 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* strain 328, incubated for 7 days at 28°C, samples were taken every 24 h. Biomass was separated by filtration and was then dried. The supernatant was used to carry out antagonism assays in solid medium and *in vivo* (Maldonado et al., 2010).

Determination of minimum inhibitory concentrations (MIC)

To measure the MIC values, two-fold serial dilutions of 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.390 and 0.187 mg ml^{-1} of the crude extract were prepared in DM solvent and assayed by well diffusion-method as described by Shahidi (2004). The MIC was defined as the lowest concentration able to inhibit any visible fungal growth. All data represent average of three replicated experiments.

Polarity detection rate of active crude extract in organic solvents

To evaluate the relative polarity of the active principle(s) present in the crude extract, 2 ml of each of H_2O, methanol and chloroform were added to 20 mg pulverized-crude samples separately and vortex for 20 min. Each sample was then centrifuged at 3000 rpm for 15 min using a bench low speed centrifuge. Supernatants and pellets were separated at 50°C and assayed at concentration of 20 mg ml^{-1} by agar diffusion-method (Bonjar, 2004).

Determination of thermal inactivation point (TIP)

Small aliquots (10 mg ml^{-1}) of soluble crude extract were exposed to 30, 40, 50, 60, 70, 80 and 90°C for 10 min and cooled on ice afterwards to monitor the effect of temperature on bioactivity. For temperatures over 90°C, heat oil was used. Bioactivity of treated samples was evaluated using well diffusion method. Control included incubation of an untreated sample at 28°C (Nawani and Kapadnis, 2004).

Detection of fungicidal activity and metabolites mode of action

Small blocks of inhibition zones (1 mm^3) of *Streptomyces* strain 328 against *P. digitatum* was transferred to fresh PDA plates and incubated for seven days at 24-28°C. During incubation, growth or lack of growth of the fungus was investigated both visually and microscopically. Rejuvenation of growth was indicative of fungistatic and lack of growth represented fungicidal properties of the antagonist.

Chloroform assay for detection of antibiotic

Spore suspensions (approximately 10^8 spores ml^{-1}) of individual isolates were dotted (10^{-6} l per spot) onto 15 ml starch casein agar plates, five dots per plate. Plates were incubated at 28°C for 3 days (Davelos et al., 2004). Dotted isolates were killed by inverting the uncovered Petri plates over 4 ml of chloroform in a Watch glass for 1 h. Watch glasses were removed, and plates were aerated in a fume hood for 30 min to permit evaporation of chloroform. Plates were subsequently overlaid with 15 ml of 1% water agar and inoculated with 10^{-6} l of test isolate, *P. digitatum*, (approximately 10^8 spores ml^{-1}) spread uniformly over the surface of the agar. Plates were incubated at 24°C for four to five days. The size of any zone of growth inhibition of the overlaid isolate surrounding any dotted isolate were measured in millimeters from the edge of the dotted colony to the edge of the cleared zone.

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*.

Scanning electronic microscopic

Mycelial morphology of *Streptomyces* strain 328, was showed by using scanning electronic microscopic (CAM SCAN – MV 2300). Preparation for the scanning electron microscope consisted of using the culture held by a piece of double stick scotch tape. The stubs were coated in a sputter coater for 2 min. Afterwards, the specimens were viewed and digital electron micrograph were prepared at magnification of 6000 to 20000x with an accelerating voltage of 20 kv accordingly.

In vivo antagonism assays

In vivo assays were carried out with *Streptomyces* strain 328 to study its inhibitory activity against *P. digitatum.* Fresh orange cultivars Valencia of uniform size and maturity, without wounds, were used in this study. They were picked and washed for 10 min in running tap water, surface sterilized in 1% sodium hypochlorite for 2 min and aseptically rinsed carefully with sterile distilled water,. Four batches of 10 oranges each were used: (1) oranges without treatment (control disease); (2) oranges treated with solution of sodium bicarbonate (200 ppm) (control without disease); (3) oranges treated with 50 ml of sodium bicarbonate (200 ppm), plus 50 ml of metabolites obtained after centrifugation (200 rpm) of *Streptomyces* culture; (4) oranges treated with 50 ml of sodium bicarbonate (200 ppm) with 50 ml of Spore of *Streptomyces* culture; (5) Oranges treated as in 3 and 4, but with the addition of 20% 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 three 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 oranges. The results reported are the average of triplicate determinations (Maldonado et al., 2010).

Experimental design and data analysis

The experiments were arranged in a completely randomized design. Each treatment was replicated three times with ten fruits for each replication. The experiments were repeated twice. Extent of disease was planned at each of the four inoculation sites by assessing presence or absence of soft rot symptoms after four day. Statistical analysis was performed with SPSS software package version 15.00 for windows. Analysis of variance and Tukey test of Post Hoc were used for analysis of recorded percentages of mean values. Significant factors in the univariate analysis at first step were entered to multivariate comparisons.

RESULTS

Antifungal bioassays

From tested *Streptomyces* isolates, eleven isolates were active in dual culture methods, showing two antagonists and the suppressive reaction of *P. digitatum*. Figure 1 shows bioassay results of *Streptomyces* strain 328 against *P.digitatum* measured in agar disk-method. In both methods *Streptomyces* strain 328 show antifungal inhibitory effects on the pathogen.

Streptomyces strain 328 in *in vitro* antagonism against several fungal strains

The *Streptomyces* strain 328 culture and its metabolites against *P. digitatum* and *P. italicum* (because *P. italicum* like *P. digitatum* produce common mold on citrus) were studied and both could inhibit fungal growth (Figure 2). Table 1 shows that the inhibitory effect was higher in the test carried out with *Streptomyces* strain 328 culture.

Monitoring antagonistic activity and growth curve

Activity reached maximum after five days in rotary cultures. In shake cultures, this interval was used to harvest cultures to prepare crude extract for use in further investigations. Activity versus post seeding time in rotary

Figure 1. Suppressive reaction of two *Streptomyces* isolates on *Penicillium digitatum*. up: *Streptomyces* isolate 328 and down: *Streptomyces* isolate 223. Center: colony of *Penicillium digitatum* agar disk which its growth towards the antagonists is inhibited clearly.

Figure 2. Bioassay results of *Streptomyces* isolates against *Penicillium digitatum* and *P. italicum*. Left; Clockwise from top: *Streptomyces* strain 328 against *P. italicum*, Center: blank agar disk (control). Right; Clockwise from top: *Streptomyces* strain 328 against *P. digitatum*, Down: blank agar disk (control).

Table 1. Antifungal activity of *Streptomyces* isolate 328 metabolites and culture against *P. digitatum* and *P. italicum.*

Penicillium spp.	Inhibition (%)	
	Streptomyces isolate 328 metabolites	*Streptomyces* isolate 328 culture
P. digitatum	20.22	70.10
P. italicum	23.70	76.55

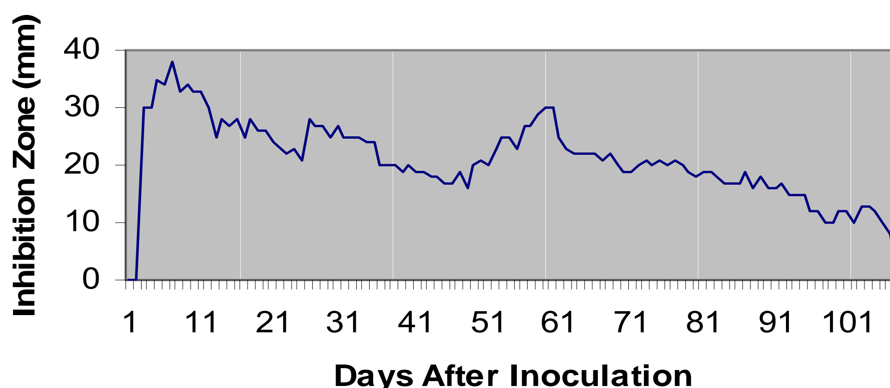

Figure 3. *In vitro* bioassay results of *Streptomyces* strain 328 against *Penicillium digitatum* in rotary cultures indicative of production time versus inhibition zones.

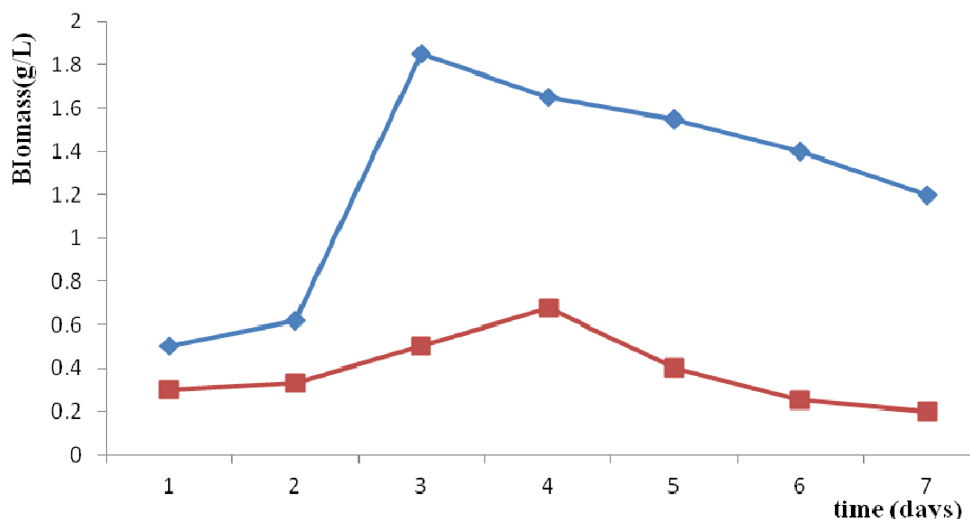

Figure 4. Growth kinetics of *Streptomyces* isolate 328 in starch casein (■) and YMG (♦) media pH 7 at 28°C during seven days.

cultures is presented in Figure 3.

***Streptomyces* strain 328 growth and metabolites production in YMG and starch casein media**

Growth kinetics for *Streptomyces* strain 328 in YMG medium showed a maximum biomass value of 1.85 g/l after 72 h incubation, while in starch casein medium the highest value was 0.68 g/l after 96 h incubation (Figure 4). The metabolites obtained from one to seven days fermentations in starch casein and YMG media were assayed against *P. digitatum* and *P. italicum* in liquid media antagonism tests. The metabolites produced in YMG after 48 to 72 h incubation showed the maximum inhibition against *P. italicum* and *P. digitatum* (54.80 and 63.27%, respectively) while those produced in starch casein presented the highest inhibition levels after 5 days fermentation (12.75 and 13.25%, respectively) as seen in Table 2. All metabolites from more than 6 days fermentation kept inhibitory activity against both phytopathogens, but their inhibition percentages were lower than the maximum values obtained in previous days.

Determination of MIC

In well diffusion-method, MIC of the crude was determined as 0.39 mg ml^{-1} against *P. digitatum* (Figure 5).

Table 2. Inhibition (%) produced by of *Streptomyces* isolate 328 metabolites from YMG and starch casein media against *P. digitatum* and *P. italicum*.

Day		Inhibition (%)						
		1	2	3	4	5	6	7
P. digitatum	Metabolites from YMG	47.22	56.12	63.27	61.30	39.03	22.41	13.70
	Metabolites from starch casein	2.5	2.75	4.75	7.25	13.25	11.65	6.21
P. italicum	Metabolites from YMG	33.5	47.25	52.03	54.80	23.5	22.01	18.69
	Metabolites from starch casein	0.36	1.83	4.02	10.50	12.75	12.33	9.67

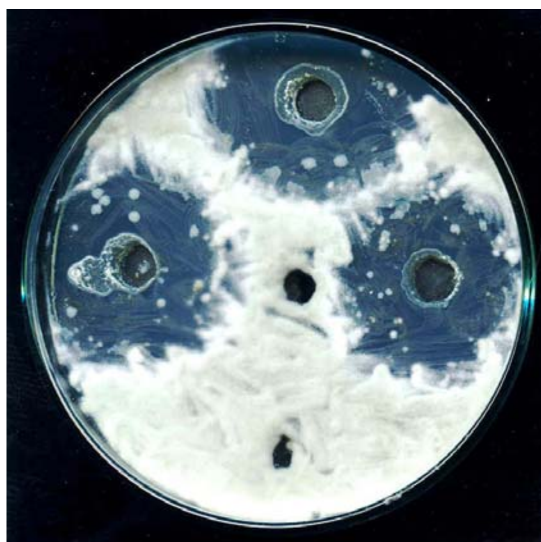

Figure 5. MIC bioassay results of *Streptomyces* isolates against *Penicillium digitatum*. Clockwise from top: *Streptomyces* strain 328 after 11 days, the *Streptomyces* after 19 days, the *Streptomyces* after 20 days, blank agar hole (control).

Polarity detection rate of active crude extract in organic solvents

Solubility results are indicated in Table 3. The results show more than one active principle involved since activity is traceable in polar solvents of H_2O, methanol.

Determination of TIP

Bioactivity of active isolate diminished to zero at 160°C.

Detection of fungicidal activity and metabolites mode of action

P. digitatum was able to grow in PDA medium after their spores were exposed to *Streptomyces* strain 328 metabolites at 28°C for five days. Based on the result we assumed that *Streptomyces* strain 328 metabolites presents fungistatic mode of action.

Table 3. Bioassay results of solubility tests of the antifungal principle(s) of *Streptomyces* isolate 328 against *Penicillium digitatum* in fractions of different solvents indicated by well diffusion-method at 10 mg ml^{-1} of dry crude.

Solvent	Fraction	Activity
H_2O	Supernatant	+
	Pellet	+
Methanol	Supernatant	+
	Pellet	+
Chloroform	Supernatant	-
	Pellet	+

Chloroform assay

Among 11 effective *Streptomyces* strain, 3 strains of 328, 263 and 394 retained their antifungal activities after exposure to chloroform.

Molecular mass evaluation for *Streptomyces* strain 328 metabolites

P. digitatum growth inhibition produced by *Streptomyces* strain 328 metabolites showed identical levels as experiential without dialysis and after this treatment. We assumed that benzoylated dialysis tube retained *Streptomyces* strain 328 metabolites and they have a molecular mass higher than 2000.

Scanning electron microscope studies

Scanning electron micrograph of mycelia of *Streptomyces* strain 328 is indicated in Figure 6 (this picture is necessary for determination of spore level and chain morphology to identification of streptomyces species).

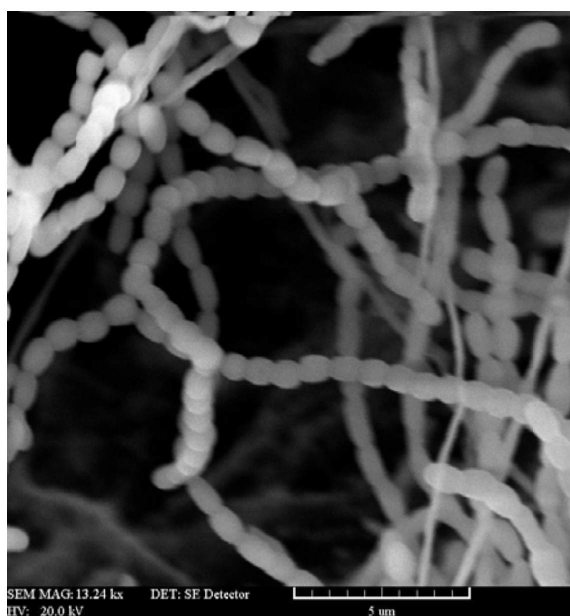

Figure 6. Scanning electron micrographs of mycelia of *Streptomyces* isolate No. 328

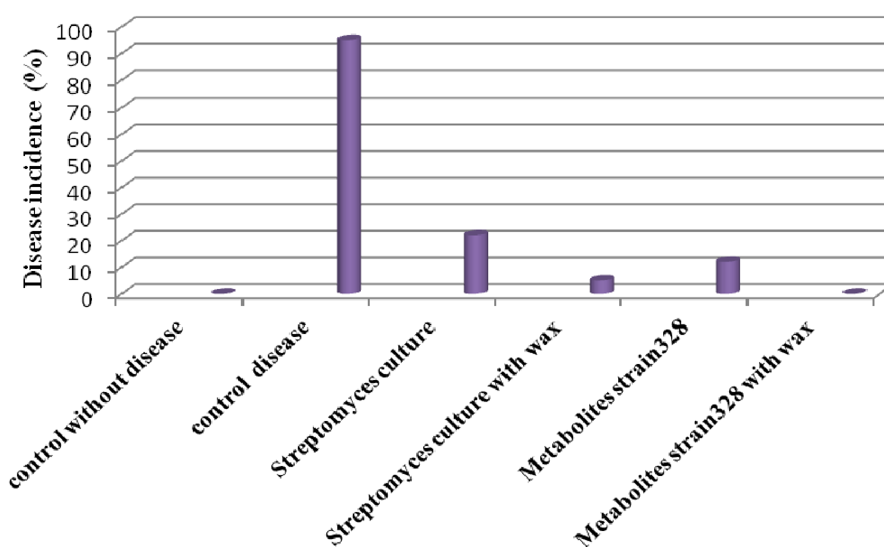

Figure 7. *In vivo* assays of *Streptomyces* isolate 328 metabolites and *Streptomyces* isolate 328 culture treated and not treated with wax against *P. digitatum*

In vivo antagonism assays

Oranges without treatment showed disease incidence percentages of 80 to 100%, while oranges with chemical fungicide treatment showed no signs of disease. When biological treatment was applied, the results were as follows: (1) *Streptomyces* culture without wax reduced disease to 22%; with wax, the disease was reduced to 5%. (2) Metabolites without wax produced disease incidence levels of 12%; with wax disease, was totally controlled (Figure 7).

Statistical data analysis

Statistical analysis of data with multiple comparisons of

Tukey test indicated 95% significance level. There was significance difference indicative of high level of antagonistic effect of *Streptomyces* strain no. 328 against *P. digitatum.*

DISCUSSION

In vitro assays of *Streptomyces* isolate no. 328 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 and Traquair (2002) and Maldonado et al. (2010); the authors also observed that maximum biomass production occurred after three days fermentation in the same conditions. The highest *Streptomyces* isolate no. 328 biomass was 1.85 g/l in YMG medium after 96 h incubation, but grew poorly in starch casein medium, which, Sabaratnam and Traquair (2002) suggested as better than other media. 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) demonstrated 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 green mold caused by *P. digitatum* was significantly reduced and wax addition contributed to fruit protection, to avoid orange dehydratation. In this case, better results were realized with *Streptomyces* isolate No. 328 metabolites, in *in vitro* assays. Similar results of disease inhibition produced by *Streptomyces* sp. R03 metabolite against lemon pathogens has been reported earlier by Maldonado et al. (2010). 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, those in the environment as the nature of its factors, interfere with other microorganisms, etc. *Streptomyces* 328 metabolites was not inhibited by heat and choloroform; this effect could be due to their chemical nature and could not inhibit the lytic enzymes activity in fermentation broth. Mahadevan and Crawford (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, β 1-3 glucanases) capable of acting on the fungus cell wall by altering growth and possibility is also common among them. *Streptomyces* isolate No. 328 metabolites have molecular mass higher than 2000 and their mode of action is fungistatic, but several authors report *Streptomyces* ability to produce fungicidal substances like Streptomycin, Kasugamycin, Polyoxin and Validamycin that have different action modes and the active ingredients of many biological plaguicides (Duran, 2004). Our findings represent the presence of potential antifungal metabolite(s) *Streptomyces* strain 328 against *P. digitatum*. Antifungal activity of the isolate found in this study highlights its importance as a candidate for further investigation in biological control of the world-wide destructive citrus postharvest disease. Further works on the project would be focused on field and long-term storage evaluation of the antagonist against the pathogen in the cosmopolitan malady of citrus postharvest disease. The genes encoding many antifungal characteristics are currently being used by agribusiness to create genetically modified plants that have increased fungal resistance in the field or packing house. We believe that the results of these findings can form the avenue for production of resistant transgenic-plants with recombinant DNA having antifungal genes cloned from biologically active *Streptomyces* isolates which would lead to environmentally safer measures in plant-pest management.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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