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Antifungal potential of *Streptomyces* sp. 3400 JX826625 ethanolic filtrate against *Penicillium digitatum*, A post-harvest spoilage agent of Citrus fruits

Andriambeloson Onja*[1], Andrianantenaina Rigobert[1], Andriamahaly Manjato Eddy[2], Ramaroson Luciano[1], Rasolomampianina Rado[1]

[1]*Centre National de Recherches sur l'Environnement, Antananarivo, Madagascar*

[2]*Faculté des Sciences, Université d'Antananarivo, Madagascar*

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Abstract

Fruit postharvest diseases, because of different losses they generate, remain a major problem affecting diverse domains in the world. Among several alternatives used to control phytopathogenic fungi, the main causal agents of fruit postharvest diseases; the efficacies of microorganism culture filtrates have been demonstrated in many works. For this purpose, this study aimed to assess the potential of *Streptomyces sp* 3400 JX826625 ethanolic filtrate to inhibit *Penicillium digitatum* growth and to control postharvest decay in citrus fruits (lemons and oranges) during the storage. *In vitro* assay using agar cylinder technique showed that the actinomycete isolate displayed antagonistic activity against *Penicillium digitatum* with an inhibition rate of 60.60±2.62%. On the other side, the ethanolic filtrate of the strain prepared from the culture on starch casein agar medium using radial growth method was very active towards the phytopathogen displaying an inhibition rate value of 77.27±4.54%. The disc technique showed an inhibition zone value of 19mm. Chemical screening of the ethanolic filtrate through precipitation and coloration assays revealed the presence of alkaloids, saponins, polyphenols, flavonoids and leucoanthocyans. *In vivo* assay with lemons and oranges presented a preventive effect of the antifungal product. An improvement of the shelf life for the two tested fruits treated with the ethanolic filtrate were recorded during artificial infection experimentation (5 days for both fruits) and storage assay (11 days for lemons and over 21 days for oranges) at ambient temperature, compared to untreated fruits of which the shelf life was 2 days (lemons) and 4 days (oranges).

* **Corresponding Author:** Andriambeloson Onja ✉ herionja@ymail.com

Introduction

As belonging to protective foods category, fruits and vegetables play a crucial role in the maintenance of human health stability. They constitute a protection source of the organism against diverse diseases due to their richness in natural products with defensive role as antioxidants, vitamins and mineral salts.

During the development at the orchard or vegetable garden, through the harvest, the transport to the storage, fruits and vegetables can endure more or less significant alterations which damages can present an important economic impact inducing a total loss of production. These alterations can be mechanical, physiological or parasitic. However, for fruits and vegetables, parasitic alterations are the most numerous, the most deleterious and the most difficult to control due to the pathogens diversity including molds, bacteria, virus and insects. Microbial alterations can be occurred at the orchard and vegetable garden by phytopathogenic microorganisms but also after harvest during their storage by spoilage microorganisms through latent infections that may occur during preharvest (Sparado and Gullino, 2004). Moreover, microbial alteration can result from mechanical damage permitting entry of the microorganisms, particularly strict wound pathogens which development induces fruit decay (Talibi *et al.*, 2014).

Indeed, phytopathogenic fungi are the principal causes of foodstuffs decay. Some of them are polyphagous, infect many species of fruits or vegetables (*Botrytis cinerea*) (Poveda *et al.*, 2020) while others are relatively particular for a type of fruit or vegetable (*Mycosphaerella fijiensis*, banana pathogen) (Carlier *et al.*, 2000). The induced diseases occasioned frequent losses of fresh products after harvest, in terms of quality and quantity. According to the FAO (2019), 20% to 40% of agricultural production in the world is damaged each year by parasites. Annual economic loss due to fungal diseases arises to several billion euros, increasing consequently the risks of famine, malnutrition and undernourishment, especially in developing countries.

The damages mostly notable are localized necrosis with mycelia development in the surface or not, intense production of spores and characteristic signs of deficiencies throughout the plant at the orchard or vegetable garden. These damages are conversely manifested by the decay of the fruits and vegetables during the storage. Besides the plants, the human can be victim of vegetal fungal attack by certain phytopathogenic fungi, producers of natural toxins dangerous for human health (patulin and citrinin, cancerous fungal toxins produced by *Penicillium*) (Dukare *et al.*, 2019).

Several controlling strategies against fungal diseases of fruits and vegetables are currently developed and reunites different domains. However, its choice depends on the objectives for obtaining best yields for the production as well as for the shelf life. As examples, the genetic control, using adapted species and varieties providing natural or induced resistance to the plants, the chemical control employing fungicides, the physical control that involves plowing, residues grinding and burying, the biological control serving bacterial, fungal parasites and botanical fungicides, the agronomic control that operates on plant cover density, irrigation, crop rotation, nitrogen fertilization and agronanotechnology focusing on the synthesis of bioactive nanoparticles from plant extracts (El-Baky and Amara, 2021).

During the storage, this control is mainly based on the regulation of the temperature, the moisture, the disinfection of the storage locals, the use of fungicides and recently the irradiation and the use of nanoparticles. Nevertheless, whatever the control adopted, the goal is to limit or to avoid the damages (symptoms, loss of yield and quality) caused by phytopathogenic fungi.

These last years, some research works conducted on the control of phytopathogenic fungi exploited the potential of natural substances especially those from plants. Extracts and essential oils were demonstrated to be efficient to control phytopathogenic fungi growth (Cobos, 2015). On the other side, biological control using antagonistic microorganisms has provided promising results. Among them, some bacteria as *Bacillus*, *Lactobacillus* and *Enterobacter* strains (Korsten, 1995; Matei *et al.*, 2015; Shi and Sun, 2017), yeasts (Pimenta *et al.*, 2010) and molds (Liu *et al.*, 2007) were used for controlling fruit postharvest pathogens.

Endophytic, telluric and rhizospheric actinobacteria were also commonly used to control pathogenic fungi in some plants (Costa, 2013; Choudhary *et al.*, 2015; Álvarez-Pérez *et al.*, 2017). However, the use of their natural metabolites to control phytopathogenic fungi infection has been less developed in the literature and different works.

Recognized as microorganisms rich in secondary metabolites with different types and biological activities including antifungal activity, the actinomycetes could develop alternatives in the phytosanitary treatment against phytopathogenic fungi. Their natural metabolites could substitute chemical substances that are concerned for the health and the environment.

Thus, this work aimed to demonstrate the effect of actinomycete antifungal metabolites against spoilage fungus development isolated from oranges (*Penicillium digitatum*) during their storage. This fungus is well-known as the causal agent of green mold, the most common and serious postharvest disease affecting citrus fruits (Talibi *et al.*, 2014). The effect of postharvest preservation of the actinomycete metabolites on two selected species of citrus fruits (oranges and lemons) are, therefore, described according to the *in vivo* antifungal test.

Materials and methods

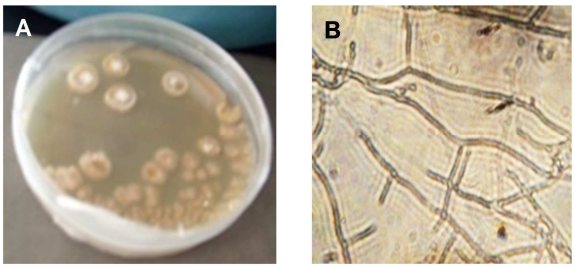
Materials

Actinomycete strain

The microorganism from which natural metabolites were extracted and used for assay in this study is an actinomycete (*Streptomyces sp* 3400 JX826625) conserved in the microbial strain collection of the Laboratory of Environmental Microbiology/National Center of Environmental Research. *Streptomyces sp* 3400 JX826625 was isolated from ginger rhizospheric soil of Andrefavinany ginger plantation (District of Soavinandriana, Region Itasy, Madagascar) and selected for its ability to inhibit numerous pathogens including fungal phytopathogens (Andriambeloson *et al.*, 2014; Andriambeloson *et al.*, 2016; Andriambeloson *et al.*, 2019).

Cultural and morphological aspects of Streptomyces sp 3400 JX826625

The selected actinomycete grown on starch casein agar (SCA) showed large (3-5mm of diameter), opaque, round and convex colonies presenting irregular contour. The aerial mycelium is white and less developed whereas the vegetative mycelium is brown, embedded in the gelose. The strain doesn't produce diffusible pigment (Fig. 1A.). Morphological characters of the actinomycete strain according to the microscopic examination revealed that *Streptomyces sp* 3400 JX826625 was characterized by a vegetative mycelium composed of long, non-fragmented, thin and branched filaments. The aerial mycelium bears about two to four rectiflexible spores chains with non-motile single spores (Fig. 1B.).



A: Cultural features of *Streptomyces sp* on SCA medium, B: Microscopic aspects of *Streptomyces sp* (40X magnification)

Fig. 1. Cultural and morphological characters of *Streptomyces* sp 3400 JX826625.

Fruit materials

Two species of citrus fruits ("Greffe" oranges, a local variety in Madagascar and lemons "*Citrus limon*") collected at fruits and vegetables commercial of Petite vitesse, Isotry (Antananarivo, Madagascar), were selected for this study. Fresh and healthy fruits without wounds, with uniform size and maturity, having received no prior antifungal treatment were chosen.

Methods

Isolation of the phytopathogenic fungus from infected fruits

The pathogen used in this study was isolated from decayed oranges covered of green mold after 3 days of storage at room temperature.

The isolation was performed using technique described by Li *et al.* (2022) with modification. The conidia from the rotted surfaces of the orange were picked with sterile loop and cultured on Sabouraud agar medium (Glucose 40g, Tryptone 10g, Agar 18g, Distilled water 1000ml) supplemented with chloramphenicol (50mg/ml) at 28°C for 5 days. The fungal colony was checked by its characteristics easily recognizable with fungal mycelia development on the culture medium.

Purification and conservation of the phytopathogenic fungus

Grown colony was purified by two or three successive subcultures onto Sabouraud agar medium exempt of antibiotic. Thereafter, pure fungal strain obtained was conserved according to two methods: the slant agar conservation for short-term preservation and the conservation at +1 to +10°C in minitubes containing sterile distilled water with a small block of mycelial agar, for long-term preservation (about 7 years) (Nguyen, 2007).

Identification of the phytopathogenic fungus

Isolated phytopathogenic fungus was identified according to Pitt's method (1988) based on its phenotypic aspects by macro and microscopic examination. Macroscopic characterization included growth rate, size, texture and color of the colony on CYA medium (Czapek Yeast Autolysate: Czapek concentrate 10ml, Sucrose 30 g, Yeast extract 5g, K_2HPO_4 1g, Trace elements stock solution 1ml, Agar 20g, Distilled water 1000ml) at different temperatures (5°C, 28°C and 37°C). On the other hand, microscopic observation was focused on conidiophore branching pattern, shape and texture of stipes, metulae/branches, phialides, conidia and cleistothecia (Pitt, 1988; Visagie *et al.*, 2014).

Microscopic examination of the target fungal strain was performed by preparing slides from a 7-10 days old *Penicillium digitatum* culture on CYA medium, mount with methylene blue stain and observed under optic microscope at 100x magnification.

Antagonism test

In vitro antagonistic activity of *Streptomyces sp* 3400 against isolated phytopathogenic fungi was evaluated using agar cylinder technique (Aghighi *et al.*, 2004). From 7 days old cultures of both germs, fungal agar cylinder (6mm) was picked using cork borer and placed in the center of the Sabouraud plate whereas two actinomycetes agar cylinders were put onto the both sides of the Petri plates at about 3cm from the fungal agar cylinder. Plates with only fungal strain were served as controls. The assay was carried out in triplicate and the cultures were incubated at 28°C for 5 days; the diameters of fungal colony towards the actinomycete agar cylinders were measured and compared with the controls. The inhibition rate of phytopathogen growth was expressed according to Kordali *et al.*'s formula (2003):

$$\text{Inhibition rate}\,(\%) = \frac{\text{D control-D test}}{\text{D control}} \text{X } 100$$

With: D control: maximal radial growth distance of the fungus

D test: radial growth distance of the fungus towards the actinomycete strain

Growth Inhibition of the phytopathogen was assessed according to a scale from 0 - 4 (Korsten *et al.*, 1995): 0 (no growth inhibition), 1 (0-25% of growth inhibition), 2 (26-50% of growth inhibition), 3 (51-75% of growth inhibition), 4 (76-100% of growth inhibition).

Antifungal activity of Streptomyces sp 3400 secondary metabolites

Filtrates and supernatants preparation

Fermentations of different liquid and solid media with *Streptomyces sp* during 8 days at 30°C were performed on AIA and AIB (Natrium caseinate 2g, Asparagine 0.1g, Natrium propionate 4g, dikalium phosphate 0.5g, Magnesium sulfate 0.1g, Iron sulfate 0.001g, Glycerol 5g, Agar 15g, Distilled water 1000ml), ISP2 Agar and ISP2 broth (Yeast extract 4g, Malt extract 10g, D-glucose 4g, Agar 20g, Distilled water 1000ml); SCA or SCB (Soluble starch 10g, Casein 1g, K_2HPO_4 0.5g, Agar 15g, Distilled water 1000ml); Bennett agar and Bennett broth (D-glucose 10g, Casaminoacids 2g, Yeast extract 1g, Meat extract 1g, Agar 15g, Distilled water 1000ml) and Sporulation agar and Sporulation broth (Yeast extract 1g, Meat extract 1g, Tryptose 2g, Fe SO_4 traces, Glucose 10g, Agar 15g, Distilled water 1000ml).

For liquid media, the actinomycete agar was inoculated in 50ml of each prepared culture medium; the cultures were then incubated at 30°C under shaking at 125rpm for 8 days. Thereafter, they were centrifuged at 10.000xg for 20mn; the supernatants were recovered and screened for antifungal assay.

For solid media, *Streptomyces sp* was grown on large Petri plates (150mm) with the five media cited above for 8 days at 30°C by streak technique using sterile swabs. Natural metabolites were then extracted by maceration of the cultures with 30ml of ethanol 95° for 2h at ambient temperature. Afterwards, ethanolic extracts were filtrated using Millipore filter (0.45µm); the obtained filtrates were tested for their antifungal activity.

Antifungal test

Antifungal activity of *Streptomyces sp* ethanolic filtrates and supernatants against *Penicillium digitatum* was assessed using radial growth method (Cobos, 2015) and disc technique. For the first method, 0.5ml of ethanolic filtrate was introduced into 20ml of molten Sabouraud agar; the mixture was homogenized manually and poured into sterile plate. After solidification, a pathogenic fungal disc (6mm in diameter) was put in the center of the plate; the test was performed in three replicates and the cultures were incubated at 28°C for 5 days. Inhibition growth percentage of phytopathogenic fungus was assessed according to the formula mentioned above. Kordali *et al.*'s criteria (2003) were taken to evaluate the activity of the filtrates and the supernatants: very active filtrate or supernatant: $75\%<x<100\%$; active: $50\%<x<75\%$; moderately active: $25\%<x<50\%$; weakly active or inactive: $0\%<x<25\%$.

Meanwhile, other filtrates were prepared and dried using a speedvac in order to determine their average concentration.

For disc method, sterile discs (6mm in diameter) soaked with 20µl of ethanolic filtrates and supernatants were put onto Sabouraud agar previously inoculated with the fungal suspension (10^6spores/ml). The cultures were incubated at 28°C for 7 days and the inhibition zone around the discs expressing the activity of the filtrates or the supernatants was measured.

Chemical screening of Streptomyces sp ethanolic filtrate

Chemical families of *Streptomyces sp* ethanolic filtrate compounds susceptible to be the active metabolites were screened using precipitation and coloration tests described by Fong *et al.* (1977). Tested families were: alkaloids, saponins,

steroids, flavonoids, leucoanthocyans, anthocyans, triterpens and polyphenols.

In vivo antifungal activity of Streptomyces sp ethanolic filtrate

Effectiveness of antifungal ethanolic filtrate prepared from *Streptomyces sp* 3400 against the selected postharvest phytopathogen was assessed in this work through *in vivo* test. Tested fruits (oranges and lemons) were inoculated with *Penicillium digitatum* and treated with ethanolic filtrate according to five assays described as follows:

- Assay 1: tested fruits were inoculated with the fungal pathogen, and then treated with *Streptomyces sp* ethanolic filtrate prepared from SCA medium.
- Assay 2: the assay 1 was repeated and tested fruits were treated with *Streptomyces sp* ethanolic filtrate after three days.
- Assay 3: tested fruits were treated with *Streptomyces sp* ethanolic filtrate and inoculated with the pathogen after 24h.
- Assay 4: tested fruits were inoculated with the fungal pathogen.
- Assay 5: tested fruits were treated with distilled water.

The experimental device is summarized in the fig. 2.

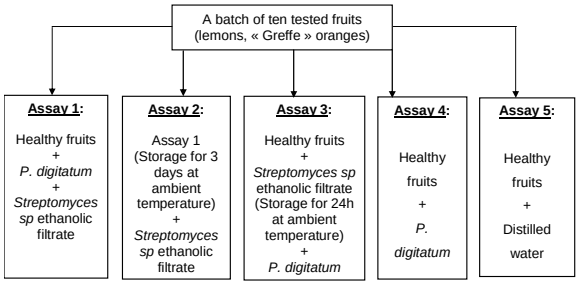


Fig. 2. Experimental device of *in vivo* assay of antifungal effect of *Streptomyces sp* 3400 JX826625 ethanolic filtrate against *P. digitatum* in lemons and "Greffe" oranges.

A batch of 10 tested fruits in three replicates was used for each assay. The fruits were surface disinfected by washing, soaking and rinsing processes with tap water, ethanol 70° for 30s, sodium hypochlorite 3% for 3min and sterile distilled water. Disinfected fruits were then placed onto plastic trays and air-dried at room temperature. A wound (about 5mm wide and 2mm deep) was made on the peel of each fruit and pathogen inoculation was performed by injection of 10µl of fungal spore suspension (10^6spores/ml) using sterile needles through wounded surface of tested fruits. For each assay, the treatment was carried out by *Streptomyces sp* ethanolic filtrate spraying of tested fruits. After dryness, a water-soaked cotton was placed into each tray to maintain the moisture, the trays were closed and left at ambient temperature (24°C) during the assay. Fungal infection manifesting by soft rot, mycelia or green mold development on the surface of the fruits was daily observed for 21 days. The disease incidence was expressed as follows (Youssef and Roberto, 2020):

$$\text{Disease incidence} = \frac{\text{Total number of infected fruits}}{\text{Total number of tested fruits}} \text{X } 100$$

Conservation test of oranges and lemons with Streptomyces sp ethanolic filtrate

This test is necessary for assessing the effectiveness of the produced ethanolic filtrate to protect tested fruits (oranges and lemons) against phytopathogens and therefore to extend their shelf life. Ethanolic filtrate was prepared from the culture of *Streptomyces sp* on SCA medium as described above. The test consisted in spraying the fruits with *Streptomyces sp* ethanolic filtrate every three days. The fruits were left at ambient temperature for 21 days, fungal mycelia development (if present) was examined and confirmed according to *Penicillium digitatum* phenotypical traits.

Statistical analysis

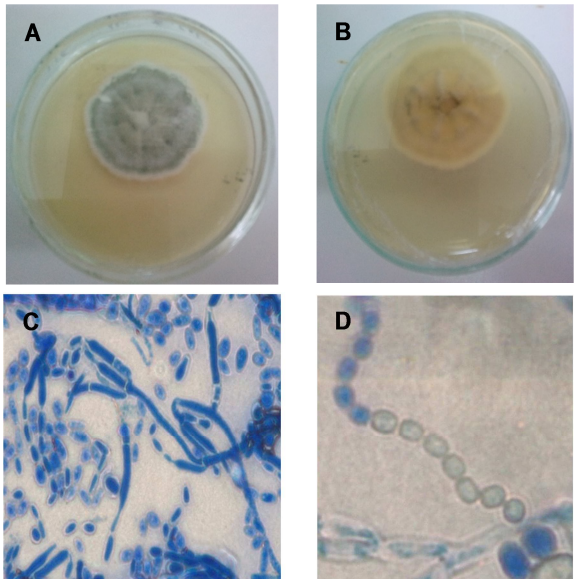
The data obtained were subjected to the analysis of variance (ANOVA). A least significant difference (LSD) test at 0.05 probability level (p=0.05) was used for means comparison.

Results

Isolation and identification of the phytopathogen

Examination of cultural and morphological features of the target phytopathogen was conducted from a one-week-old pure colony grown on CYA medium. Macroscopically, the fungal strain showed moderate growth and dull green colony with 35mm of diameter at 28°C. Fungal growth was recorded at 5°C whereas no growth was observed at 37°C. The fungus presented granular powdery texture and didn't produce exudate or soluble pigment. The back side of the colony was yellow orange.

On the other hand, microscopic aspects revealed aerial hyphae bearing irregular branched conidiophores. The stipes presented terveticillate structure, the phialides were cylindrical and solitary. The conidia were ellipsoid to cylindrical with smooth wall and arranged in long chains (Fig. 3.).



A: Front side of *Penicillium digitatum* on CYA medium, B: Reverse side of *Penicillium digitatum* on CYA medium, C: Conidiophore branching pattern of *Penicillium digitatum*, D: Conidia of *Penicillium digitatum*

Fig. 3. Macro and microscopic features of *Penicillium digitatum* isolated from orange green mold.

In comparison with *Penicillium digitatum* characteristics described by Pitt (1988), Domsch (1993) and Li *et al.* (2022), the isolated strain is confirmed and identified as *Penicillium digitatum*.

In vitro antagonism of Streptomyces sp 3400

Antagonistic activity of the isolate

Preliminary screening of *Streptomyces sp* 3400 for its antagonistic activity against citrus fungal pathogen using agar cylinder technique showed that the actinomycete strain displayed antagonistic activity against *Penicillium digitatum* with an inhibition rate of 60.60±2.62% (Fig. 4A.) categorized in Korsten *et al.*'s scale 3.

Antifungal activity of Streptomyces sp 3400 secondary metabolites

According to the results obtained, the filtrates and supernatants from *Streptomyces sp* culture on different solid and liquid culture media exhibited activity against *Penicillium digitatum*, except AIA and AIB media on which no growth of the tested actinomycete was recorded. It would be also emphasized that the activity of the filtrates and the supernatants varied from one medium to another. However, this activity was weak for the supernatants compared to that recorded for the ethanolic filtrates. The ethanolic filtrate prepared from the culture of *Streptomyces sp* 3400 on SCA medium, at a concentration of 536±31.08µg/ml was very active towards *Penicillium digitatum*, displaying the highest inhibition rate value of 77.27±4.54% (Table 1, Fig. 4B.).

Similarly, the disc technique confirms its antifungal activity against *Penicillium digitatum* with an inhibition zone evaluated at 19mm (Table 1, Fig. 4C.).

Chemical screening of Streptomyces sp ethanolic filtrate

The results of chemical screening shown in the table 2, revealed that alkaloids, saponins, polyphenols, flavonoids and leucoanthocyans were present in the SCA ethanolic filtrate.

Table 1. Inhibition rate of *Penicillium digitatum* by *Streptomyces sp* ethanolic filtrates and supernatants.

	Inhibition rate (%)				Inhibition zone (mm)			
	Bennett	ISP2	SC	Sporulation	Bennett	ISP2	SC	Sporulation
Ethanolic filtrates	48.48±5.24^b	19.69±2.62^b	77.27±4.54^b	37.87±2.62^b	10±0.57^b	9.25±0.5^b	19^b	10.75±0.28^b
Supernatants	9.87±2.14^a	11.9±4.12^a	29.30±1.72^a	21.42±3.39^a	7.83±0.28^a	7.17±0.28^a	11±1^a	9^a

The data in the same column followed by the same letter don't show significant difference according to Anova test (p=0.05)

A: Antagonistic activity by cylinder agar method (center: *P. digitatum*; top and bottom: *Streptomyces sp*), B: Antifungal activity by radial growth method (left: control; right: assay), C: Antifungal activity by disc method

Fig. 4. *In vitro* antagonistic and antifungal activities of *Streptomyces sp* isolate and *Streptomyces sp* ethanolic filtrate against *Penicillium digitatum*.

Table 2. Chemical screening of *Streptomyces sp* ethanolic filtrate components.

Chemical families	Tests	Observation (positive reactions)	*Streptomyces* sp ethanolic filtrate
Alkaloids	Mayer	Precipitation	+
	Wagner	Precipitation	+
	Dragendorff	Precipitation	+
Flavonoids	Willstätter	Red coloring, purple red, purplish red	+
	Willstätter modified	Red coloring, purple red, purplish red	+
Leucoanthocyanes	Bate-Smith	Red purplish coloring	+
Anthocyanes	Borntrager	Red	-
Triterpenes	Liebermann Burchard	Pink	-
Steroides	Salkowski	Red separation ring	-
	Badjet-Kedde	Red	-
Anthraquinones	Borntrager	Red	-
Saponins	Foam height	Persistence of foam height for 30 min.	+
Polyphenols	Gelatine 1%	Precipitation	+
Tanins	Salted Gelatine	Precipitation	-
	$FeCl_3$	Black- blue coloring, green-black, bluish-black	-
Polysaccharides	-	Precipitation	-

-: negative, +: positive

In vivo antifungal activity of Streptomyces sp ethanolic filtrate

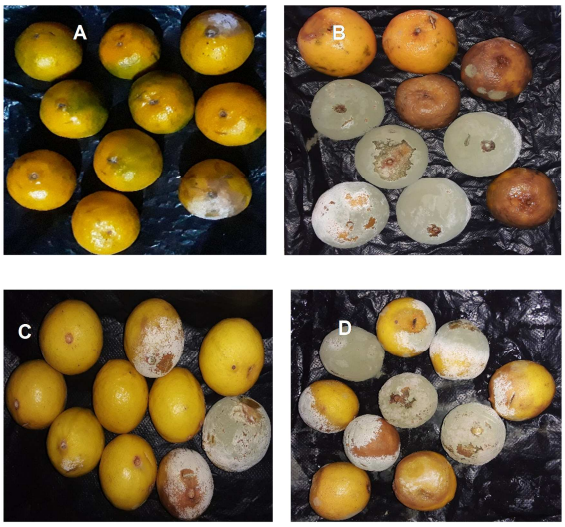
Through the results obtained, it would be deduced that *in vivo* assay confirmed the results of *in vitro* assay. For the test with lemons, compared to the controls (treatments with the pathogen and distilled water), a reduction of disease incidence was recorded when the fruits were treated with the ethanolic filtrate. The best results were obtained for the batch treated with the ethanolic filtrate followed by the inoculation of *Penicillium digitatum* after 24h. A significant extension of storage time (5 days) was noted, implying the remarkable preventive effect of the

ethanolic filtrate. With the oranges, the same tendency of results was observed. However, the infection rate of *Penicillium digitatum* was weaker than that on lemons. The results obtained were similar for the treatment with ethanolic filtrate sprayed every three days and that with the ethanolic filtrate applied on the oranges before pathogen inoculation, 80% of the oranges were not infected until the 21st day (Table 3, Fig. 5).

Table 3. Disease incidence recorded in lemons and oranges according to the treatments with *Streptomyces sp* ethanolic filtrate.

	Disease incidence (%)					
	Lemons			Oranges		
	4 days	6 days	14 days	6 days	14 days	21 days
Assay 1	56.67 ± 5.77^{c}	100^{b}	100^{b}	$36.67\pm5.77^{bc,c}$	$33.33\pm11.54^{a,b,\ ab}$	$40^{\ ab,b,c}$
Assay 2	36.67 ± 5.77^{b}	56.67 ± 5.77^{a}	56.67 ± 5.77^{a}	$13.33\pm5.77^{ab,b}$	$20^{a,ab}$	$20^{\ ab,b,}$
Assay 3	0^{a}	60^{a}	60^{a}	0^{a}	16.67 ± 5.77^{a}	$20^{\ ab,b,}$
Assay 4	100^{d}	100^{b}	100^{b}	53.33 ± 11.54^{c}	$100^{b,c,bc}$	$100^{\ bc,c,d,}$
Assay 5	73.33 ± 11.54^{c}	100^{b}	100^{b}	46.67 ± 5.77^{c}	$100^{b,c,bc}$	$100^{\ bc,c,d,}$

The data in the same column followed by the same letter don't show significant difference according to Anova test (p=0.05)



A: "Greffe" oranges treated with the ethanolic filtrate and inoculated with *P. digitatum* after 24h (observation after 14 days), B: "Greffe" oranges treated with *P. digitatum* (observation after 14 days), C: lemons treated with the ethanolic filtrate and inoculated with *P. digitatum* after 24h (observation after 6 days), D: lemons treated with *P. digitatum* (observation after 6 days)

Fig. 5. *In vivo* antifungal activity of *Streptomyces sp* ethanolic filtrate on oranges and lemons.

Conservation assay

For the lemons, no signs of deterioration was observed after 11 days of treatment with the ethanolic filtrate every three days. Twenty percent (20%) of the lemons were infected from the 12th day and 40% were healthy on the 21st day. It was observed that fruits deterioration was started by soft rot followed by fungal mycelia development. Untreated lemons showed, however, decays on the 3rd day of storage.

Concerning the conservation assay of the oranges, no decay was noted during 21 days of observation. Nevertheless, some physical changes as shrinking and color change (brown) of the fruits was observed at the end of the experimentation. The inner of the fruits after peeling kept healthy and fleshy orange pulps. About untreated oranges, the occurrence of green mold was recorded on the 6th day of observation.

Discussion

This work aims to search natural metabolites from microorganisms able to inhibit *Penicillium digitatum* development in citrus fruits after harvest. In this case, *Streptomyces sp* 3400JX826625, an actinomycete from ginger rhizospheric soil was selected and the efficiency of its natural metabolites to inhibit target phytopathogen growth both *in vitro* and *in vivo* was demonstrated, in order to establish postharvest treatment for preventing green mold development on citrus fruits during the storage.

The phytopathogenic fungus was isolated from the oranges infected by green mold. Examination of its macroscopic and microscopic features showed characters relatively close to those of *Penicillium digitatum* (growth at 5°C, terveticillate structure of conidiophore, ellipsoidal conidia in chain) and allowed to confirm the isolated fungus belonging to this specie. This fungus is reported in the literature as a common plant pathogenic fungus causing postharvest disease especially green mold in citrus but some works demonstrated also its ability to inhabit other hosts as nectarine, plum, pome and stone fruit (Louw and Korsten, 2019), leading to devastating disease propagation and consequently, heavy losses in fruit crops .

In vitro antagonism assay of *Streptomyces sp* against isolated phytopathogenic fungus using agar cylinder technique showed that the actinomycete exerted fungal development inhibition with Korsten *et al*.'s scale estimated at 3. Thus, the use of *Streptomyces sp* 3400 JX826625 in the biocontrol against green mold development in citrus fruits is conceivable. In the other side, the potential of *Streptomyces sp* natural metabolites to inhibit phytopathogenic fungus development was assessed. All ethanolic filtrates and supernatants prepared from different culture media (Bennett agar, ISP2 agar, SCA, Sporulation agar) inhibited *Penicillium digitatum* development. However, the inhibition rate varied from one media to another and ethanolic filtrates showed strong activity than supernatants. The highest inhibition rate was recorded with the ethanolic filtrate prepared from *Streptomyces sp* cultured on SCA medium. Hence, the activity expressed by *Streptomyces sp* isolate was lower than *Streptomyces sp* metabolites implying that the actinomycete strain exerted antifungal activity against *Penicillium digitatum* by antibiosis mechanism.

Jose *et al.* (2013), Bhosale *et al.* (2015) emphasized that the composition of culture media constitutes an important factor for extrolites synthesis in microorganisms. As in plants, they can act as natural fungicides for controlling phytopathogenic fungi and include phenolics, fatty acids, flavonoids, alkaloids, glycosides, terpenoids, and tannins (El-Baky and Amara, 2021). As reported by Masoko and Eloff (2005), various antifungal compounds mixed together will possibly work in synergistic manner against phytopathogenic fungi. Thus, several antifungal metabolites in synergistic action could be produced by the actinomycete strain from the different components of the SCA medium and inhibited the pathogen growth.

Indeed, chemical screening of the SCA ethanolic filtrate revealed the presence of alkaloids, flavonoids, saponins, polyphenols and leucoanthocyanes. Remarkable antifungal properties of these metabolites against many phytopathogenic fungi were highlighted in numerous works.

The flavonoids isolated from *Mangifera indica* leaves showed interesting antifungal effect on *Alternaria alternata*, *Aspergillus fumigatus*, *Aspergillus niger*, *Macrophomina phaseolina* et *Penicillium citrii* (Kanwal, 2010). Those from *Asteriscus graveolens* were active towards *Botrytis cinerea*, *Penicillium digitatum* and *Penicillium expansum* (Alilou *et al.*, 2016).

Porsche *et al.* (2018) supported in their work the antifungal activity of saponins extracted from *Sapindus mukorossi* fruit pericarps on *Venturia inaequalis* and *Botrytis cinerea.*

Likewise, the antifungal activity of alkaloïds was demonstrated in some investigations against *Exserohilum turcicum, Bipolaris maydis, Alternaria solani, Sclerotinia sclerotiorum, Fusarium oxysporum* (Zhang *et al.*, 2009) as well as on some saprophytic and pathogenic fungi such as *Alternaria alternata*, *A. solani*, *A. brassicicola*, *A. brassicae*, *Curvularia lunata*, *C. pallescens*, *C. maculans*, *Curvularia species*,

Colletotrichum species, *C. musae*, *C. gloeosporioides*, *Erysiphe pisi*, *Fusarium udum*, *Helminthosporium echinoclova*, *H. pennisetti*, *H. spiciferum* and *Heterosporium* sp (Singh, 2007).

Moreover, Aguirre-Joya *et al.* (2018) reported in their study the antifungal properties of polyphenols against plant pathogenic fungi including *Botrytis cinerea, Alternaria alternata, Colletotrichum gloeosporioides* and *Fusarium oxysporum*.

From these findings it would be deduced that the antifungal activity of *Streptomyces sp* 3400 JX826625 ethanolic filtrate from a culture on SCA medium could be due to the activities of the identified chemical components in the ethanolic filtrate.

In vivo assay confirmed the pathogenicity of *Penicillium digitatum* which caused green mold in the two citrus fruits tested (lemons and oranges). According to the results obtained, it was noted that the severity of the postharvest disease varied according to the citrus fruits species. *Penicillium digitatum* was more aggressive in lemons than in oranges, disease occurrence and infection rate were respectively, earlier and higher in lemons than in oranges for all assays. This may be due to the difference of fruit peels composition (native microorganisms, secondary metabolites) which contribute to their protection against pathogens.

The treatment of citrus fruits with *Streptomyces sp* ethanolic filtrate allowed to reduce the risks of fungal affection by *Penicillium digitatum*. However, the efficiency of the treatment varied from one fruit specie to another and from one treatment to another. It was noted that the tested filtrate didn't present a curative effect on the infected fruits. In despite of this, repetitive applications (every three days) and application of the ethanolic filtrate prior to the inoculation of the fungal phytopathogen allowed the reduction of disease incidence in the two tested fruits and the extension of fruits conservation (5 days for both fruits) compared to the control and the treatment with one application of the filtrate during the storage. These findings are in accordance with several works for the development of strategies on the control of citrus fruits postharvest decay. Our results related to those of Abraham *et al.* (2010) for their investigation on the biocontrol of *Penicillium digitatum* using some yeast and *Bacillus* isolates as biocontrol agents. They demonstrated that the application of the biocontrol agents 3h and 48h before the inoculation of the phytopathogen reduced its growth on the surface of the lemons and Valencia oranges, prevented their decay and reduced infection incidence inferior to 5% on Valencia oranges. The authors reported also that the biocontrol agents didn't produce a curative action when the application of the biocontrol agent were post-infection.

Wound protection control against this strict wound phytopathogen by the ethanolic filtrate showed efficacy displaying an enhancement of storage times for the two tested fruits. Wounded fruits were protected against phytopathogen infection during 5 days for both fruits. These results are comparable to those found by Perez *et al.* (2016), for their work on the ability of some yeasts to control postharvest fungal diseases in lemons. The yeast strains 27, 28 and 56 allowed to protect the wounds against *Penicillium digitatum* infection with efficiencies rates of 93.3%, 82.5% and 72.5%, respectively within 5 days at 24°C.

Moreover, certain investigations conducted on the capacity of biocontrol agent supernatants or culture filtrates from liquid media to act on the inhibition of the phytopathogen suggested their suitability for controlling green mold in citrus. Najmeh *et al.* (2014) demonstrated that metabolites from *Streptomyces* strain 328 reduced better oranges disease incidence than *Streptomyces* culture. Likewise, Chen *et al*. (2018)

confirmed the potential inhibition effect of *Bacillus amyloliquefaciens* DH-4 culture filtrate against *Penicillium digitatum* to control citrus green mold.

In *in vivo* assays, application methods play an important role on the efficacy of plant extracts for controlling postharvest diseases in citrus fruits (Talibi, 2014). Several methods such as coating, dipping, spraying are the most practical used and the application time varied from one method to another. In this study, repetitive sprays showed promising results in the case of oranges and lemons prevention against green mold during artificial infection assay as well as in storage assay. A significant reduction of *Penicillium digitatum* infection was remarked in lemons during the experimentation and an extension of the shelf life for the two tested fruits was recorded compared to the untreated fruits for storage assay. As the most of biocontrol agents or bioactive products (plant essences or extracts) tested for their effect against *Penicillium digitatum*, *Streptomyces sp* 3400 JX826625 ethanolic filtrate presented also preventive effect.

Conclusion

This work allowed to highlight the preventive effect of *Streptomyces sp* 3400 JX826625 ethanolic filtrate prepared from the strain culture on SCA medium against *Penicillium digitatum* development and decay in lemons and oranges during the storage. Thus, this research suggests the product as a promising alternative for controlling citrus postharvest disease caused by *Penicillium digitatum*. Otherwise, further studies will be necessary to better understand the inhibition mechanism of the ethanolic filtrate against *Penicillium digitatum* growth, to assess its postharvest effect when the treatment will be performed before the harvest at the orchard and to identify the active principles that comprise the ethanolic filtrate.

References

Abraham AO, Laing MD, Bower JP. 2010. Isolation and in vivo screening of yeast and *Bacillus* antagonists for the control of *Penicillium digitatum* of citrus fruit. Biological Control **53,** 32-38.

Aghighi S, Shahidi Bonjar GH, Rawashdeh R, Batayneh S, Saadoun I. 2004. First report of antifungal spectra of activity of Iranian actinomycetes strains against *Alternaria solani*, *Alternaria alternata*, *Fusarium solani*, *Phytophtora megasperma*, *Verticillium dahlia* and *Saccharomyces cerevisiae*. Asian Journal of Plant Sciences **4,** 463-471.

Aguirre-Joya JA, Pastrana-Castro L, Nieto-Oropeza D, Ventura-Sobrevilla J, Rojas-Molina R, Aguilar CN. 2018. The physicochemical, antifungal and antioxidant properties of a mixed polyphenol based bioactive film. Heliyon **4,** 1-14.

Alilou H, Bencharki B, Talbi J, Barka N. 2016. Activite Antifongique Des Flavonoïdes Isolés De La Plante *Asteriscus Graveolens Subsp. Odorus (Schousb.) Greuter*. European Scientific Journal **12 (12),** 258-269.

Álvarez-Pérez JM, González-García S, Cobos R, Olego MA, Ibañez A, Díez-Galán A, Garzón-Jimeno E , Coque JJR. 2017. Use of Endophytic and Rhizosphere Actinobacteria from Grapevine Plants To Reduce Nursery Fungal Graft Infections That Lead to Young Grapevine Decline. Applied and Environmental Microbiology **83 (24),** 1-16.

Andriambeloson HO, Rafalisoa BL, Andrianantenaina R, Rasamindrakotroka AJ, Rasolomampianiana R. 2019. Effect of *Streptomyces sp* 3400 JX826625 Metabolites on Multidrug Resistant *Candida albicans* Development and Chemical Characterization of Antifungal Metabolites. American Journal of Biomedical and Life Sciences **7(6),** 164-173.

Andriambeloson HO, Rasolomampianina R, Ralambondrahety R, Andrianantenaina R, Raherimandimby M, Randriamiharisoa F. 2016. Biological Potentials of Ginger Associated *Streptomyces* Compared with Ginger Essential Oil. American Journal of Life Sciences **4(6)**, 152-163.

Andriambeloson O, **Rasolomampianina R, Raherimandimby M.** 2014. Selection and characterization of bioactive actinomycetes associated with the medicinal plant Ginger (*Zingiber officinale*). Journal of International Academic Research for Multidisciplinary **2(9),** 30-45.

Bhosale HJ, Kadam TA, Fulwad SG, Karale MA, Kanse OS. 2015. Optimization of antifungal compound production by a moderately halophilic *Streptomyces werraensis* HB-11. International journal of pharmaceutical sciences and research **6(3),** 1190-99.

Carlier J, Zapater MF, Lapeyre F, Jones DR, Mourichon X. 2000. Septoria leaf spot of banana: a newly discovered disease caused by *Mycosphaerella eumusae* (anamorph Septoria eumusae). Phytopathology **90(8),** 774-890.

Chen K, Tian Z, Luo Y, Cheng Y, Long C. 2018. Antagonistic Activity and the Mechanism of *Bacillus amyloliquefaciens* DH-4 Against Citrus Green Mold. Phytopathology **108,** 1253-1262.

Choudhary B, Nagpure A, Gupta RK. 2015. Biological control of toxigenic citrus and papaya-rotting fungi by *Streptomyces violascens* MT7 and its extracellular metabolites. Journal of Basic Microbiology **55 (12),** 1343-1356.

Cobos R, Mateos RM, Álvarez-Pérez JM, Olego MA, Sevillano S, González-García S, Garzón-Jimeno E, Coque JJR. 2015. Effectiveness of Natural Antifungal Compounds in Controlling Infection by Grapevine Trunk Disease Pathogens through Pruning Wounds. Applied and Environmental Microbiology **81(18),** 6474-6483.

Costa FG, Zucchi TD, Soares de Melo I. 2013. Biological Control of Phytopathogenic Fungi by Endophytic Actinomycetes Isolated from Maize (*Zea mays* L.). Brazilian archives of biology and technology **56(6),** 948-955.

Domsch KH, Gams W, Anderson TH. 1993. Compendium of soil fungi. Vol. I & II, reprint IHW - Verlag. Eching, Germany, 859 + 405 p.

Dukare AS, Paul S, Nambi VE , Gupta RK, Singh R, Sharma K, Vishwakarma RK . 2019. Exploitation of microbial antagonists for the control of postharvest diseases of fruits: A review. Critical Review in Food Science and Nutrition **59,** 1498–1513.

El-Baky NA, Amara AAAF. 2021. Recent Approaches towards Control of Fungal Diseases in Plants: An Updated Review. Journal of fungi **7(11),** 900.

FAO. 2019. De nouvelles normes visent à freiner la propagation mondiale des parasites et des maladies nuisibles aux plantes. Communiqué de presse, AllAfrica.

Fong HHS, Tin WAM, Farnsworth NR. 1977. Phytochemical screening. Review. Chicago: University of Illinois 73-126.

Jose PA, Sivakala KK, Jebakumar SRD. 2013. Formulation and Statistical optimization of culture medium for improved production of antimicrobial compound by *Streptomyces sp* JAJ06. International Journal of Microbiology 1-9.

Kanwal Q, Hussain I, Siddiqui HL, Javaid A. 2010. Antifungal activity of flavonoids isolated from mango (*Mangifera indica* L.) leaves. Natural Product Research **24(20),** 1907-1914.

Kordali S, Cakir A, Zengin H, Duru ME. 2003. Antifungical activities of the leaves of three *Pistacia* species grown in Turkey. Fitoterapia **74(1-2),** 64-167.

Korsten L, De Jager E, De Villiers EE, Lourens A, Kotzé JM, Wehner FC. 1995. Evaluation of bacterial epiphytes isolated from avocado leaf and fruit surfaces for biocontrol of avocado postharvest diseases. Plant Disease **79,** 1149-1156.

Li Y, Xia M, He P, Yang Q, Wu Y, He P, Ahmed A, Li X, Wang Y, Munir S, He Y. 2022. Developing *Penicillium digitatum* Management Strategies on Post-Harvest Citrus Fruits with Metabolic Components and Colonization of *Bacillus subtilis* L1-21. Journal of fungi **8(80),** 1-17.

Liu X, Wang J , Gou P, Mao C, Zhu ZR, Li H. 2007. In vitro inhibition of postharvest pathogens of fruit and control of gray mold of strawberry and green mold of citrus by aureobasidin A. International Journal of Food Microbiology **119,** 223–229.

Louw JP, Korsten L. 2019. Impact of Postharvest Storage on the Infection and Colonization of *Penicillium digitatum* and *Penicillium expansum* on Nectarine. Plant Disease **103(7),** 1584-1594.

Masoko P, Eloff JN. 2005. The diversity of antifungal compounds of six South African *Terminalia* species (Combretaceae) determined by bioautography. African Journal of Biotechnology **4 (12),** 1425–1431.

Matei A, Cornea CP, Matei S, Matei GM, Rodino S. 2015. Comparative antifungal effect of lactic acid bacteria strains on *Penicillium digitatum*. Bulletin UASVM Food Science and Technology **72 (2),** 226–230.

Najmeh S, Hosein SBG, Sareh S, Bonjar LS. 2014. Biological control of citrus green mould, *Penicillium digitatum*, by antifungal activities of Streptomyces isolates from agricultural soils. African Journal of Microbiology Research **8 (14),** 1501-1509.

Nguyen MT. 2007. Identification des espèces de moisissures, potentiellement productrices de mycotoxines dans le riz commercialisé dans cinq provinces de la région centrale du Vietnam - Étude des conditions pouvant réduire la production des mycotoxines. Thèse de doctorat, Université de Toulouse, France 147p.

Perez MF, Contreras L, Garnica NM, Fernández-Zenoff MV, Farías ME, Sepulveda M, Ramallo J, Dib JR. 2016. Native killer yeasts as biocontrol agents of postharvest fungal diseases in lemons. PLOS ONE 1-21.

Pimenta RS, Silva JFM, Coelho CM, Morais PB, Rosa CA, Corrêa A Jr. 2010. Integrated control of Penicillium digitatum by the predacious yeast *Saccharomycopsis crataegensis* and sodium bicarbonate on oranges. Brazilian Journal of Microbiology **41,** 404-410.

Pitt JI. 1988. A laboratory guide to common *Penicillium* species (2nd ed.). Commonw Scientif Ind Research Organisation, North Ride Australia 197p.

Porsche FM, Molitor D, Beyer M, Charton S, André C, Kollar A. 2018. Antifungal Activity of Saponins from the Fruit Pericarp of *Sapindus mukorossi* against *Venturia inaequalis* and *Botrytis cinerea*. Plant disease **102,** 991-1000.

Poveda J, Barquero M, González-Andrés F. 2020. Insight into the Microbiological Control Strategies against *Botrytis cinerea* Using Systemic Plant Resistance Activation. Agronomy **10(11),** 1-19.

Shi JF, Sun CQ. 2017. Isolation, identification, and biocontrol of Antagonistic *bacterium* against *Botrytis cinerea* after tomato harvest. Brazilian Journal of Microbiology **48(4),** 706–714.

Singh AK, Pandey MB, Singh UP. 2007. Antifungal Activity of an Alkaloid Allosecurinine against Some Fungi. Mycobiology **35(2),** 62-64.

Sparado D, Gullino ML. 2004. State of the art and future prospects of the biological control of postharvest fruit disease. International Journal of Food Microbiology **91(2),** 185-194.

Talibi I, Boubaker H, Boudyach EH, Ait Ben Aoumar A. 2014. Alternative methods for the control of postharvest citrus diseases. Journal of Applied Microbiology **117,** 1-17.

Visagie CM, Houbraken J, Frisvad JC, Hong SB, Klaassen CHW, Perrone G, Seifert KA, Varga J, Yaguchi T, Samson RA. 2014. Identification and nomenclature of the genus *Penicillium*. Studies in Mycology **78,** 343-371.

Youssef K, Roberto SR. 2020. Premature Apple Fruit Drop: Associated Fungal Species and Attempted Management Solutions. Horticulturae **6 (31),** 1-10.

Zhang JW, Gao JM, Xu T, Zhang XC, Ma YT, Jarussophon S, Konishi Y. 2009. Antifungal Activity of Alkaloids from the Seeds of *Chimonanthus praecox*. Biochemistry and biodiversity **6(6),** 838-845.