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RESEARCH ARTICLE

Antifungal activity of bacteria isolated from palm wine against postharvest rot fungi on yam (*Dioscorea* spp.)

Patrice Kouamé Assiri[1,*], Séka koutoua[1], Hortense Atta. Diallo[1], and Sévérin Aké[2]

1. Université Nangui Abrogoua/UFR-SN, Laboratoire de Biologie et Amélioration des Productions Végétales, 02 BP 801 Abidjan 02, Côte d'Ivoire

2. Université Felix Houphouet Boigny/UFR-Biosciences, Laboratoire de Physiologie Végétale, 22 BP 582 Abidjan 22, Côte d'Ivoire

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Corresponding Author

Patrice Kouamé Assiri

Abstract

One hundred twenty bacteria isolated from palm wine sample extracted from palm oil trees (*Elaeis guineensis* Jacq.) were tested against *Penicillium oxalicum* Currie and Thom and *Aspergillus niger* van Tiegh, yam (*Dioscorea* spp.) postharvest rots fungi. Inhibitory activity against at least one fungus was detected for 14 bacteria, *in vitro* and *in vivo* on yam discs. Some of the bacteria liquid culture filtrates showed and effectiveness on the two fungi *in vitro*. However, any filtrate was effective on protecting yam against *A. niger* growth and also against its spore germination.

The effectiveness of the bacteria culture filtrates was influenced by the heat and the high dilution fold up to 1000.

INTRODUCTION

Yam (*Dioscorea* spp.) is the second most important tuber in Africa after cassava (FAOSTAT, 2003). More than 90 % of the total world production of all cultivated yam species takes place in West Africa (Morse et al., 2000). Yam is an important source of carbohydrate for many people of the sub-Sahara region, especially in the yam zone of West Africa (Akissoé et al., 2003) and serves as an important source of income (Babajide et al., 2006).

Postharvest losses in yam have been attributed to insects, nematodes, rodents, respiration of the dormant tuber, loss of water by evaporation, sprouting and microbial attack (Okigbo, 2005). Microbial pathogens, especially fungi, are regarded as the main cause of tuber loss during storage (Morse et al., 2000). Many fungal pathogens have been found to infect yam tuber (Okigbo, 2002). The most important include *Botryodiplodia theobromae* Pat., *Penicillium oxalicum* Currie and Thom; *P. sclerotigenum* Yamamoto, *Fusarium moniliforme var. subglutinans* Wollens and Reinking, *Aspergillus niger* Van Tiegh, *A. tamari* kita and *Rhizoctonia* spp. (Okigbo, 2005). Yam rot usually starts in the soil and progresses during storage, although the infected tubers initially do not display any signs of external symptoms (Okigbo and Ogbonnaya, 2006).

Diverse chemical pesticides have been used in the control of postharvest spoilage of yam, including sodium, Orthophenylphenate, Borax, Captan, Thiobendazole, Benomyl, local gin, lime (Okigbo, 2004). However, such method has the disadvantage of environmental pollution, accumulation in the ecosystem and induction of pesticides resistance (Okigbo and Ikediugwu, 2000). Furthermore, chemical pesticides are costly and require expertise in the safe handling by the farmers.

To manage these problems, there is a growing trend towards the search for alternatives to chemical pesticides in many crops (Bailey and Lazorovits, 2003). Previous studies have reported that some bacteria can serve as excellent biological control agent against soil-borne pathogens (Uppal et al., 2007). Generally, bacterial

biocontrol agents work through the production of antibiotics and siderophores for nutrient competition (Lugtenberg et al., 2003), niche exclusion and/or indirectly through the induction of acquired host resistance (Uppal et al., 2007).

Palm wine is an alcoholic beverage produced by tapping the sap of the oil palm tree (*Elaeis guineensis* Jacq.). It constitutes a good growth medium for numerous microorganisms especially yeast and bacteria (Bechem et al., 2007).

In the present study, bacteria isolated from palm wine was investigated for their ability to control postharvest rot of yam (*Dioscorea* spp.) caused by different fungi.

Materials and methods

Biological materials

In this study, the most important postharvest spoilage fungi: *P. oxalicum* Currie and Thom, *A. niger* Van Tiegh, *Rhizoctonia* sp., *Fusarium* sp., *Sclerotium* sp. and *Curvularia* sp. were used. They were isolated from yam rots obtained from storage sites and markets in Abidjan (Côte d'Ivoire). All fungi were cultivated on Potato Dextrose Agar (PDA, Difco) at room temperature (25 to 30 °C). Palm wine used for bacteria isolation was extracted from palm oil tree in Côte d'Ivoire, collected according the method of Obire (2005) and stored at -80 °C until used.

Isolation of bacteria

One hundred µl aliquots of palm wine were taken aseptically and pipetted in 10 ml of a solution containing 50 g glucose, 10 g yeast extract 170 mg/ml cycloheximide, 1000 ml distilled water and 4 g potassium dihydrogen phosphate. After 2 days incubation at 28 °C under day light the suspension obtained was diluted to 10^{-3} to 10^{-5} and 100 µl were plated on solid medium containing 50 g glucose, 10 g yeast extract, 12 g bacto-Agar, 1000 ml distilled water. The different Petri dishes were incubated at 28 °C for 3 days. Colonies obtained were subcultured by streaking repeatedly on agar medium until pure cultures were obtained. All isolates were examined according to colony color, cell morphology and diameter.

Preparation of liquid culture filtrates of bacteria

Bacteria were grown in 250 ml Erlenmeyer flasks containing 100 ml of liquid medium previously described and incubated for 3 days at 28 °C in shaker at 110 rpm under day light. Cultures media were centrifuged at 3200 rpm for 20 min with a Beckman GS-6R centrifuge. The supernatants were collected and sterilized by filtration through 0.2 µm Millipore filters. The sterilized filtrates were collected and used for the experiment.

Pathogenicity test

Yam tubers without any visible signs of rotting were first washed in a running tap water and sterilized by blazing 70 % alcohol. Small discs of about 15 mm diameter and 10 mm thickness were punched out of the yam tubers. Three discs were placed on a glass microscope slide in a Petri dish containing wet filter paper. Three Petri dishes were used for each fungus to be tested. Each yam disc was inoculated with 5 µl of spore suspension at 10^6 spores/ml of the respective fungi and incubated at 28 °C under day light. Seven days after incubation, symptoms were evaluated on all discs. The test was conducted three times

In vitro antifungal activity tests

Six 8 mm diameter mycelial plugs obtained from well sporulating 7-day-old colonies were placed in sterile water, shaken and centrifuged at 250 rpm for 15 min. The supernatants were collected and the concentrations of the suspension were determined using a hematocytometer and adjusted to 10^6 spores/ml. Five hundred µl of spore suspension were plated on solid medium in Petri dishes. Plates were incubated for 24 h at 28 °C. Then, sterile filter paper discs whatman (6 mm diameter) were placed on the Agar surface and 5 µl of bacterial suspension (10^6 bacteria/ml) or liquid culture filtrate to be tested were pipetted on the discs. Control experiments were set up without the addition of any bacteria suspensions or filtrates but were just treated with sterile liquid medium previously described. Three replicate plates were prepared for each treatment. After incubation for 4 days at 28 °C under day light, the inhibitory diameters around the discs were measured. This experiment was repeated three times.

In vivo antifungal activity tests

Yam discs obtained as previously described, were soaked in bacteria suspension (10^6 bacteria/ml) or liquid culture filtrates for 3 min and then left to dry for 5 min under the sterile hood before being inoculated with 5 µl of spore suspension (10^6 spore/ml). Three discs were put on a glass microscope slide by Petri plates containing wet filter paper. Control yams discs were soaked in liquid medium before inoculation with the spore suspension.

This experiment was conducted separately for each bacterial suspension and liquid culture filtrate. After 7 days of incubation at 28 °C under day light, the severity of the symptoms was evaluated on a 0 to 3 scale.
0: no reaction of the tissue,
1: reaction of the tissue and/or low growth of the fungus at the inoculation zone,
2: growth of the fungus and invasion of the tissue,
3: sporulation of the inoculum.

Spore germination assay
Germination of spores was analyzed by mixing 100 µl of spore suspension with an equal volume of liquid culture filtrate and grown in 12 well multiwell plates (Nuncleon Delta). Controls were set up in the same manner, with liquid medium in place of liquid culture filtrate. The plates were incubated at 28 °C for 36 hours and the spore growth steps were estimated under a light microscope (Nikon eclipse E 800) using the following scale
0: no germination,
1: development of the germ tube shorter than the spore diameter,
2: development of the germ tube equal to the spore diameter,
3: development of the germ tube longer than the spore diameter.
Percentages were calculated from data obtained.

Effect of heat and dilution on liquid culture filtrate effectiveness
To evaluate the effect of heat treatment on liquid culture filtrates, filtrate samples were exposed to 40, 60, 80 and 100 °C in a water bath for one hour before being tested for their antifungal activity.
Dilution effect on liquid culture was performed by diluting the various filtrates obtained 10, 100 and 1000 fold, before testing then for their antifungal activity.

Data analysis
Each type bacteria inhibitory diameter was measured in two different directions of each disc on the *in vitro* test for each fungus and the mean was considered as the inhibitory diameter of the bacteria. Data from bacteria inhibitory diameter and symptom severity on yam disc were subject to analysis of variance using general linear model procedure, Tukey's honestly significant difference (Tukey HSD) mean comparison. The statistic software R 2.9.0 was used.

Results

Pathogenicity test
The pathogenicity tests carried out on yam discs using *Aspergillus niger*, *Penicillium oxalicum*, *Sclerotium rolfsii*, *Curvularia* sp., *Fusarium* sp. and *Rhizoctonia* sp. revealed that only *Aspergillus niger*, *Penicillium oxalicum* and *Fusarium* sp. induced rot in yam discs. However, *P. oxalicum* and *A. niger* induced the most important rot compared to *Fusarium* sp. (Fig 1). Those two fungi were therefore selected for further experiment. *P. oxalicum* produced green conidia on the yam surface while *A. niger* conidia were blackish. *Sclerotium rolfsii*, *Curvularia* sp. and *Rhizoctonia* sp. did not caused yam rot.

***In vitro* inhibitory effect of bacteria and bacterial culture filtrates**
A total of 120 bacterial isolates were isolated from palm wine samples. They were screened for their antifungal activity against *A. niger* and *P. oxalicum*. Inhibitory activity against at least one fungus was detected for 14 isolates. Among those bacteria, 8 inhibited the growth of *A. niger* (Fig 2) while 6 isolates exhibited antifungal activity against *P. oxalicum* (Fig 3). Three of the 14 bacteria isolates inhibited the growth of both fungi, namely isolates b1, b36 and b58. All the bacteria isolates induced inhibitory diameters larger than 12 mm against the two fungi although there was a certain variation in the size of the inhibitory diameter depending on the bacterial isolate as well as on the fungal strain. The isolates b4 and b58 induced the largest inhibitory diameter against *P. oxalicum* growth (14 mm in diameter) whereas against *A niger*, isolates b7, b89 and b58 displayed the most prominent effect with inhibitory diameter up to 15 mm in diameter.
The 14 bacterial isolates selected were tested for antifungal activity of their culture filtrates obtained from their growth in liquid medium for 3 days. All liquid culture filtrates tested inhibited growth of *A. niger* mycelia (Table 1). However, only 4 filtrates inhibited the growth of *P. oxalicum* (Fig 4). The inhibitory diameters created by the filtrates were smaller than those caused by the bacteria themselves. They ranged from 9 to 10 mm (Table 2).

Assessment of the ability of bacteria and bacterial culture filtrates to protect yam against fungi

In the *in vitro* tests carried out on agar plates, 14 bacteria were selected for their antifungal activity against either *P. oxalicum* or *A. niger* mycelial growth. These 14 bacteria were tested for their ability to protect yam against *P. oxalicum or A. niger*. Fig 5 and 6, respectively, show the effect of a pre-treatment with these bacteria on the spread of *P. oxalicum* and *A. niger* in yam discs. Yam discs that had not been soaked in bacterial suspension (control) were totally covered by the fungus. Compared with the control, all 6 bacterial isolates showed an antifungal activity against *P. oxalicum*. Two types of inhibition were observed: isolates b1 and b4 completely suppressed the growth of the fungi on the yam discs whereas with isolates b2, b5, b36 and b58 a slower growth rate was observed (Fig 5). Only isolates b1, b10, b36 and b58 reduced the mycelial growth of *A. niger*, (Fig 6). Isolates b1, b36 and b58 controlled the growth of both *A. niger* and *P. oxalicum* on the yam discs.

This study revealed 3 effects induced by the bacteria. On one hand, the suppression of the fungus growth, then a small growth and on the other hand no effect on the growth.

Similar tests carried out with culture filtrates on yam discs showed the ineffectiveness of all the filtrates against *A. niger* growth. However, 4 filtrates (b1, b4, b36 and b58) induced protection in yam against *P. oxalicum* (Fig 7).

Effect of culture filtrates on the germination of *A. niger* and *P. oxalicum* spores

One hundred percents of spores germination was observed with 8 filtrates (b1, b7, b8, b10, b89, b36, b47 and b58) tested against *A. niger* spores. Contrary to *A. niger*, *P. oxalicum* spore germination rates were significantly reduced by 4 filtrates (b1, b4, b89 and b58). Their inhibition rate varied from 44 to 75 %. A comparison of the different filtrates showed that b58 was the more efficient in inhibiting spore germination of *P. oxalicum* (Fig 8). On the control for which spores were untreated (without any filtrates), germination rate reached 72 % against 70 % for b2 which recorded the higher germination rate among all treatments.

Effect of heat and concentration on culture filtrates efficacy

These tests were only carried out with *P. oxalicum* since no antifungal activity was detected with bacterial filtrates on *A. niger* growth. The activity of the 4 filtrates that inhibited *P. oxalicum* growth persisted for up to 40 °C. The inhibitory diameter ranged from 8 to 10 mm. Beyond this temperature no antifungal activity was detected.

Table 3 shows that no significant difference was observed for 0, 10 and 100 fold dilution of the bacterial filtrates concerning their inhibitory activity on *P. oxalicum* growth. in contrast, no antifungal activity was detected at 1000-fold dilution.

Table 1. Inhibition of *Aspergillus niger* growth by bacterial suspensions and bacterial culture filtrates.

	Inhibitory diameter (mm)	
Bacteria isolates	Bacterial suspension	Culture filtrate
b1	12 ± 0.53 a	0
b7	14 ± 0.34 a	0
b8	13 ± 0.23 a	0
b10	13 ± 0.46 a	0
b89	12 ± 0.22 a	0
b36	14 ± 0.65 a	0
b47	13 ± 1.34 a	0
b58	14 ± 1.17 a	0
control	0 b	0

Values are the means of three replicates. Means in column followed by the same letter are not significantly different according to Tukey's honestly significant difference test at $p \leq 0.05$.

Table 2. Inhibition of *Penicillium oxalicum* growth by bacteria suspension and bacteria liquid culture filtrates.

	Inhibitory diameter (mm)	
Bacteria isolates	Bacterial suspension	Culture filtrates
b1	12 ± 0.84 a	10 ± 0.48 a
b2	13 ± 1.21 a	0 b
b4	15 ± 0.92 a	9 ± 0.82 a
b5	13 ± 1.34 a	0 b
b36	12 ± 1.13 a	10 ± 0.93 a
b58	15 ± 0.67 a	9 ± 1.45 a
control	0 b	0 b

Values are the means of three replicates. Means in column followed by the same letter are not significantly different according to Tukey's honestly significant difference test at $p \leq 0.05$.

Table 3. Effect of bacteria liquid culture filtrates at different dilution fold on *P. oxalicum* growth.

	Inhibitory diameter (mm)			
Bacteria filtrates	A	B	C	D
b1	10 ± 0.47 a	10 ± 0.94 a	9 ± 1.18 a	0 b
b4	9 ± 0.62 a	8 ± 1.06 a	8 ± 0.23 a	0 b
b36	8 ± 0.89 a	8 ± 0.31 a	8 ± 0.49 a	0 b
b58	10 ± 0.17 a	9 ± 0.46 a	9 ± 0.31 a	0 b

A: 0-fold dilution; B: 10-fold dilution A; C: 100-fold dilution; D: 1000-fold dilution .Values are the means of three replicates. Means in row followed by the same letter are not significantly different according to Tukey's honestly significant difference test at $p \leq 0.05$.

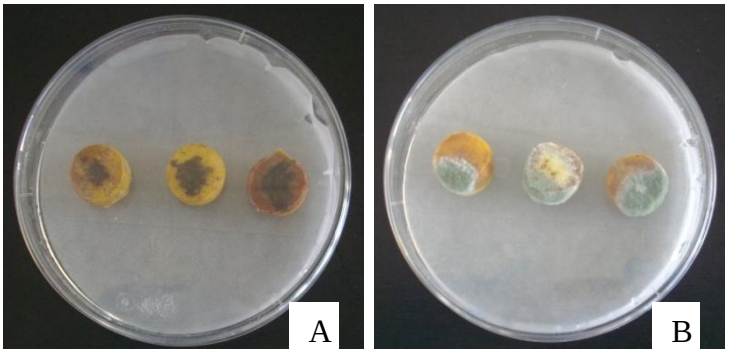

Fig 1. Fungus growth in healthy yam discs inoculated with fungus spore suspension (10^6 spores/ml). (A) *Aspergillus niger*, (B) *Penicillium oxalicum.*

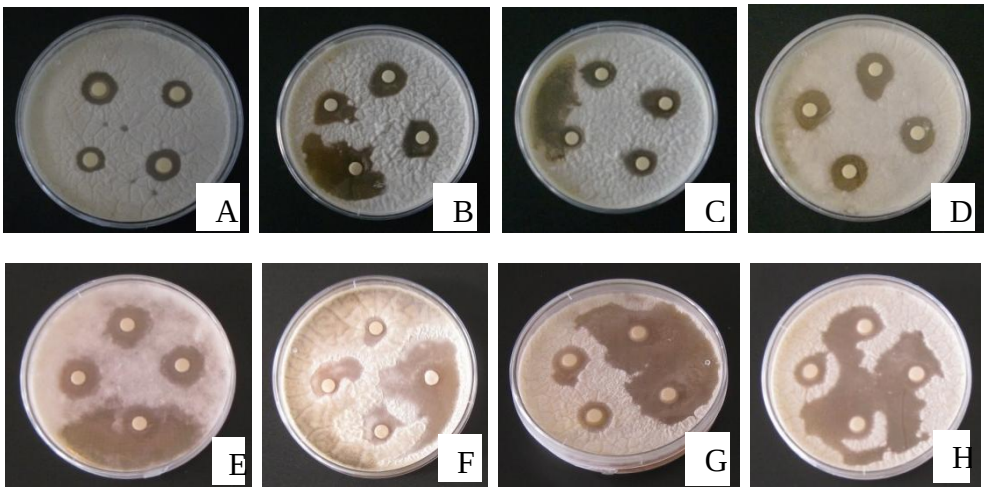

Fig 2. Effect of various bacteria on growth of *Aspergillus niger*.
(A) b1, (B) b7, (C) b8, (D) 10, (E) b58, (F) b89, (G) b36, (H) b47

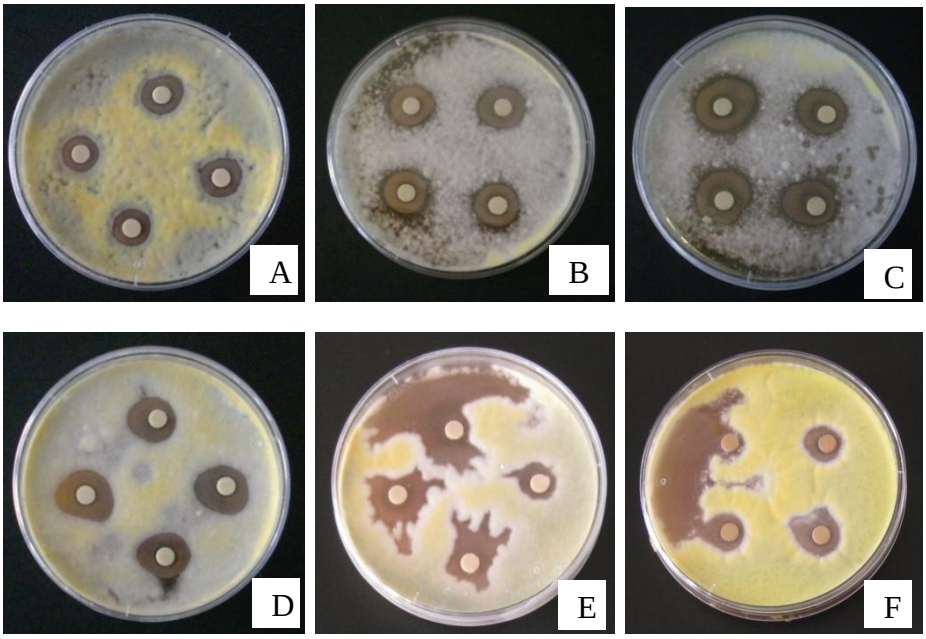

Fig 3. Effect of various bacteria on growth of *Penicillium oxalicum*.

(A) b1, (B) b2, (C) b4, (D) b5, (E) b58, (F) b36

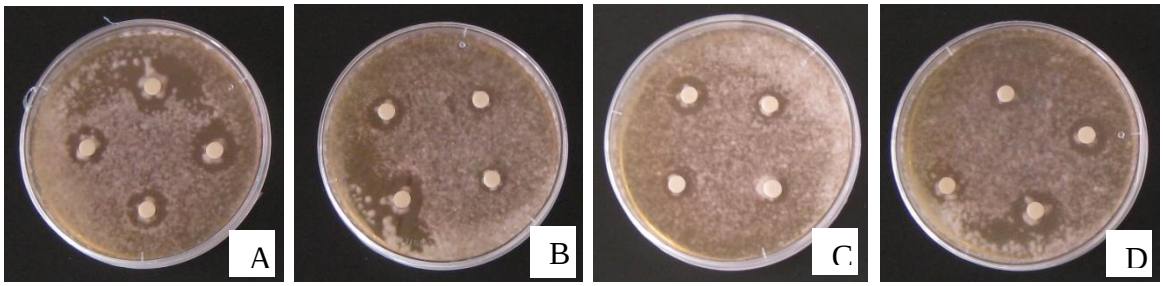

Fig 4. Effect of bacteria liquid culture filtrates on growth of *Penicillium oxalicum*.

(A) b8, (B) b1, (C) b36, (D) b47, (E) b36, (F) b58, (G) b4, (H) b7

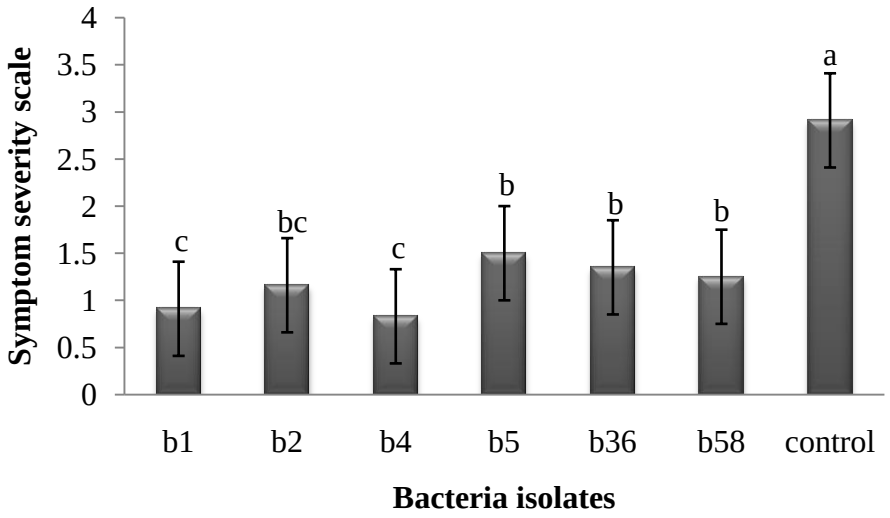

Fig 5. Antifungal activity of bacteria against *P. oxalicum* on yam disc.

Values are the means of three replicates. Means followed by the same letter are not significantly different according to Tukey's honestly significant difference test at $p \leq 0.05$.

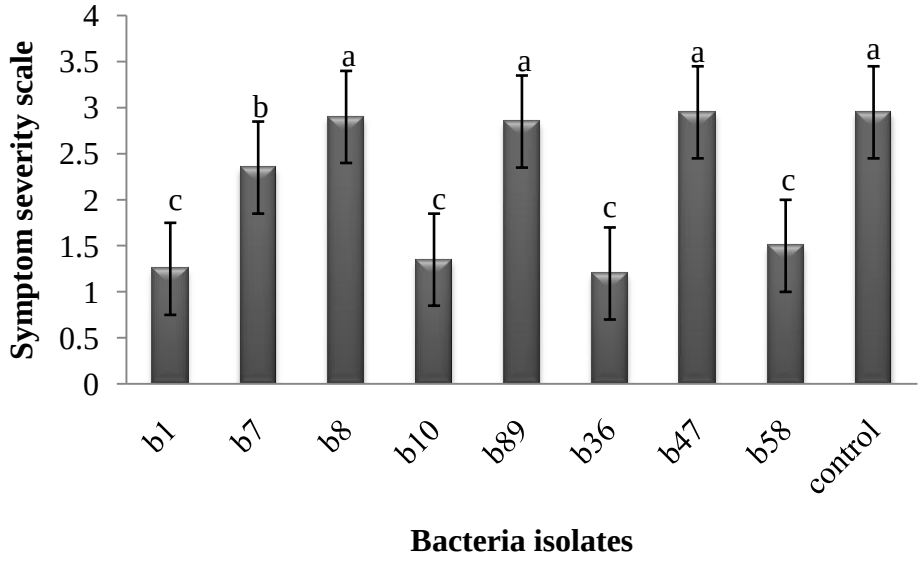

Fig 6. Antifungal activity of bacteria against *A. niger* on yam disc.

Values are the means of three replicates. Means followed by the same letter are not significantly different according to Tukey's honestly significant difference test at $p \leq 0.05$.

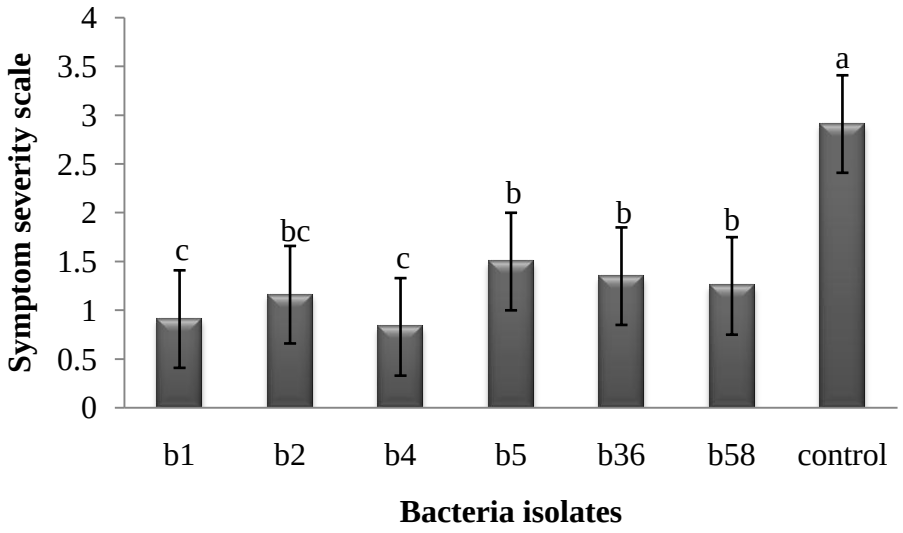

Fig 7. Antifungal activity of bacteria filtrates against *P. oxalicum* on yam disc. Values are the means of three replicates. Means in column followed by the same letter are not significantly different according to Tukey's honestly significant difference test at $p \leq 0.05$.

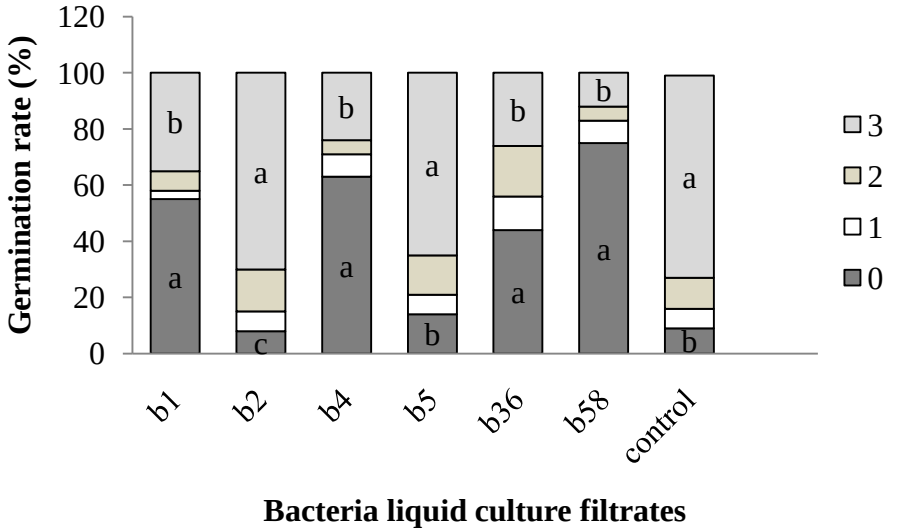

Fig 8. Bacteria filtrates inhibitory activity on *P. oxalicum* spores germination.
0, 1, 2, 3 : spores germination steps. 0: no germination, 1: development of the germ tube shorter than the spore diameter, 2: development of the germ tube equal to the spore diameter, 3: development of the germ tube longer than the spore diameter. Values are the means of three replicates. Means in column followed by the same letter are not significantly different according to Tukey's honestly significant difference test at $p \leq 0.05$.

Discussion

Pathogenicity test carried out in this study revealed that *Penicillium oxalicum* and *Aspergillus niger* induced the most important rot in yams, indicating that these microorganisms are implicated in yam spoilage. This finding was similar to that of Yusuf and Okusanya (2008) who observed *A. niger* and *P. oxalicum* among the important rot pathogens associated with stored and marked yam tubers. Microorganisms responsible for rot in tubers have long been studied and identified by several authors (Okigbo and Ikediugwu, 2000; Okigbo and Nmeka, 2005). These fungi include the ones that were used in this study.

Fourteen bacteria out of 120 screened showed fungicidal properties against either *P. oxalicum* or *A. niger*, yam postharvest fungi. Several authors showed the ability of many microorganisms such as bacteria in controlling postharvest fungi. Thus, Okigbo in 2005 showed that a strain of *Bacillus subtilis* strongly inhibited the mycelia growth of *A. niger*, *P. oxalicum* and *Botryodiplodia theobromae* for up to 60 %. Swain et al. (2008) also indicated the inhibitory activity of *B. subtilis* on many yam postharvest fungi. This observation is similar to those of Gupta et al. (2007) who indicated an inhibitory effect on growth of several black mold fungi isolated from mangrove. In their study, they obtained inhibitory diameters of about 8 mm. In 2008, Uppal et al. observed that the antagonistic activity of these microorganisms can be due to different mechanisms such as antibiotic production, competition for micronutrients or parasitism by enzyme production.

The same test carried out with filtrates of those bacteria showed that on *P. oxalicum* growth, some of the filtrates exhibited antifungal activity. In contrast to result obtained on *P. oxalicum*, no filtrates led to inhibitory activity on *A. niger* growth. This result suggests that inhibitory growth of *A. niger* by bacteria could have been due to faster nutrient uptake by those bacteria. The current result also supports the hypothesis that the effectiveness of bacteria filtrates on *P. oxalicum* could have been due to antibiotic production by those bacteria. *Bacillus*, *Streptomyces* and *Stenotrophomonas* produce compounds such as oligomycin A, kanosamin, zwittermicin A or laxanthobacin which interfere with the sporulation and the growth of broad spectrum of fungus (Raaijmakers, 2002). *Bacillus subtilis* has been found to have broad suppressive properties for more than 23 types of plant pathogen *in vitro* due to its ability to produce antibiotic (Stein, 2005).

All bacteria which inhibited *A. niger* and *P. oxalicum* mycelial growth *in vitro* did not product the same effect on yams. On one hand, some of the bacteria suppressed completely or partially their growth, inducing a small growth of the mycelia. A similar result was obtained by Mascher and Defago (2000) with bacteria isolated from palm wine. In their study, 3 types of antifungal activities against yam rot fungi were observed. First, the inhibition of growth, then the inhibition of sporulation and finally the inhibition of both growth and sporulation. Okigbo in 2002 showed that after treatment of yam tuber with *B. subtilis* and storage for five months, only 16 % developed a rot due to fungi. Additionally, no inhibitory effect was observed with the others bacteria.

Treatment with some bacteria filtrates effectively suppressed the development of *P. oxalicum* in yam, as opposed to *A. niger*. This result confirm the fact that those bacteria product some antifungical compound which are efficient in protecting yam against *P. oxalicum* but not against *A. niger*.

None of the bacteria filtrates inhibited *A. niger* spores germination. However, four filtrates were effective in inhibiting spores germination of *P. oxalicum*. It appears that the inhibitory compound secreted by bacteria in the filtrates may have specific activity against *P. oxalicum*. These results indicate that filtrates obtained with those bacteria can inhibit the spore of *P. oxalicum*, one of the most important yams postharvest fungi. Our results also indicate that the inhibitory effect of those bacteria filtrates differs depending on the fungi and the origin of the filtrates. Okigbo in 2005 observed a 100 % inhibition of the spore of *P. oxalicum*, *A. niger* and *Fusarium solani* with undiluted culture filtrates of *B. subtilis.* He suggested that this antifungal activity could have been due to iturin, an antifungal substance identified from *B. subtilis* and reported to inhibited spores germination of many fungi.

Bacteria culture filtrates under increasing temperature treatment, effect remained active against *P. oxalicum* growth for up to 40 °C. This result suggests that the active compound produced by the bacteria in the filtrates could have been proteins and also heat-labile. Most of the time heat induced protein denaturation involving a change on its structure with the loss of its biological activity (Mohacsi-farkas et al., 1999).

Our results shown that, the bacteria filtrates lost their effectiveness when the dilution fold is very high. The current result supports the hypothesis that, the susceptibility of *P. oxalicum* could depend on the concentration of filtrates produced by these bacteria. A similar result was obtained by Georgia et al. (2009) with essential oil and its major compound on the growth of *Aspergillus flavus* and *Aspergillus parasiticus*.

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