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Highlighting *Bacillus subtilis* GA1 antifungi potentialities for pineapple (*Ananas comosus*) conservation in Côte d'Ivoire

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Abstract

Pests, microorganisms and parasites are responsible for significant losses crops and especially fruits and vegetables, which threaten food human. Côte d'ivoire, the first provider of pineapple (*Ananas comosus*) fresh on European market is facing in recent years to a drastic drop in production to several factors including the action of microorganisms during storage. The struggle Chemical although effective drawbacks. This study aims using the *Bacillus subtilis* strain GA1 in biological control against germs responsible for alteration pineapple fruit in côte d'ivoire. A sample of twenty-five pineapple which has been used five healthy pineapple and five altered were used for the isolation of microorganisms and fifteen healthy pineapples were used for other tests. The main agents Fungal spoilage isolated pineapple fruit in this work were *Aspergillus* sp., *Rhizopus* sp., *Fusarium* sp., And *Candida* sp. The pathogenicity tests also confirmed that the isolated fungal strains are responsible for the pineapple fruit rotting. The tests antagonists conducted in the presence of *B. subtilis* GA1 against fungi isolated showed inhibition rate of 81.2% for *Aspergillus* sp (s), 69% for *Aspergillus* sp (a), 64% for *Rhizopus* sp., and 57.14% for *Fusarium* sp. protection tests on fruits from biomass of *B. subtilis* GA1 helped preserve fruits over a period of fourteen (14) days with no mushrooms in the heart of the fruit.

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Introduction

First provider of pineapple (*Ananas comosus*) fresh on European market (78 000 t), Côte d'Ivoire is facing to enormous post- harvest losses. Treatment with synthetic fungicides is the primary means of reducing post-harvest losses estimated to 50% worldwide (Wilson *et al.* 1993). However, the development of fungicide-resistant strains of pathogens, the detection of undesirable chemical residues in the food chain and the deregistration of some of the most effective fungicides have intensified the search for safer approaches to efficiently control post-harvest decay caused by microbial infections (Wilson *et al.,* 1991; El-Ghaouth, 1997). Among the alternatives, biological control through the use of natural antagonistic microorganisms has been extensively studied and some yeast, fungal and bacterial strains have been shown to be effective against various post-harvest pathogens (Wisniewski and Wilson, 1992; Pusey *et al.,* 1993). Several strains belonging to the genus *Bacillus* and particularly to *B. subtilis* and the closely related *B. amyloliquefaciens* species were reported effective for the biocontrol of multiple plant diseases caused by soil born microorganisms. (Asaka and Shoda, 1996; Chen and Wu, 1999; Harris and Adkins, 1999) or post-harvest pathogens (Ferreira *et al.,* 1991; Sholberg *et al.,* 1995; Mari *et al.,* 1996).

Antibiotic production by some bacteria plays a major role in disease suppression (Raaijmakers *et al.,* 2002). So far, Gram-negative bacteria, especially Pseudomonas strains, have been intensively investigated with regard to the production of antimicrobial metabolites (Keel *et al.*, 1990; Thomashow *et al.,* 1990; Howell *et al.,* 1993; Whipps, 2001). However Gram-positive bacteria and especially strains of *Bacillus subtilis* also produce a variety of antibacterial and antifungal antibiotics such as zwittermicin-A (He *et al.,* 1994), kanosamine (Stabb *et al.,* 1994) and lipopeptides from the surfactin, iturin and fengycin families.

Several strains of *B. subtilis* of various origins were isolated in Bioindustry laboratory on the basis of antibiotic production. When used as seed treatment, some of them were shown to alleviate seedling diseases presumably through direct antibiosis against the soil borne pathogen (unpublished results).

In this work, targeted at isolating and identifying fungal pathogens responsible for post-harvest rot of pineapple one particular *B. Subtilis* strain named GA1 was first tested for its ability to antagonize *in vitro* the growth of a wide variety of plant pathogenic fungi. *B.subtilis*GA1 was further studied for its potential reduces to disease of pineapple caused by the fungi during post-harvest storage.

Material and methods

Bacterial strain

B. subtilis GA1 provided from the Collection of Bioindustry Unit to University of Liège Gembloux agro biotech (Belgium). Strains were storage in cryotubes in presence of 20 % of glycerol at -80°C.

Isolation and phenotypic pathogen identification

Diseased pineapple fruits were swabbed in 70% ethanol for 2 min rinsed in two changes of sterile distilled water and the blotted dry with sterile filter papers. Necrotic lesions were aseptically cut, plated on sterile potato dextrose agar (Merck, Germany) and incubated at 28±1°C (Ewekeye *et al.*, 2013).Pure cultures were obtained by several transfers of colony growth from PDA plates to clean PDA plates aseptically. Isolates were identified based on the growth patterns, color of mycelia and microscopic examination of vegetative and reproductive structures according to Botton *et al.* (1990).

In vitro antagonism experiments

Bacillus subtilis GA1 were examined for its inhibitory effect against isolated fungi which was obtained from diseased pineapple. *In vitro* antifungal activity was assessed according to the method of Korsten and Jager (1995**)**. Briefly, strains were cultured diametrically on Potato Dextrose Agar (PDA) and one mm-diameter mycelia disk of a pure culture of the pathogen were spotted at each part of the strains of *Bacillus subtilis*. As control, a disk of the pathogen was placed at the center of PDA medium in a plate.

Plates were incubated for one week at 26°C in darkness and the radius of each fungal growth was measured. Relative growth inhibitions were expressed as a percentage. The experiment was conducted twice.

Inhibition rate= $\left[\frac{(R-r)}{R}\right]$ x100 (Oktay and Kemal, 2010)

r: Radius of microorganism growth in presence of *Bacillus subtilis* GA1

R : Radius of microorganism growth without treatment

Post-harvest disease reduction by strain GA1

Mature pineapple fruits (variety MD-2) used in all experiments was carefully selected without disease or wounding symptoms. Fruits surface were disinfected by dipping ethanol 70% for 3 min, rinsed three times with sterile distilled water and dried under filter-sterilized air flow. Then six millimetre wide and 3 mm deep wells were then artificially created with a sterile cork borer. Fruits were treated with the bacterial antagonist by adding 50 µl of cell or endospore suspension containing either 10^5, 10^6, 10^7 endospores /ml depending on the experiment, in each wounded site 24 hours prior to pathogen challenge. Infection with pathogen was realized in all cases 24 hours after treatment with *Bacillus,* by adding the same volume of a conidial suspension prepared as described above in order to introduce 10^5 conidia per site. Treated fruits were incubated in a laminar air-flow cabinet at 22°C and disease incidence was evaluated 6, 15 and 21 days after pathogen challenge based on the diameter of spreading grey mould lesions that developed around infected sites.

Microbial inocula preparation for in vivo assays

Bacterial biomass suspensions used in biocontrol experiments were prepared from 72h old cultures of *B. subtilis* GA1 grown at 30° C in agitated flasks (105 rpm) in 500 ml of a culture 863 medium (yeast-peptone-dextrose). Cultures were centrifuged at 35 000 g for 10 min and the biomass pellet was washed twice in sterile saline water (0.85 % NaCl) and stored at 4°C.

Test of fruit protection

Fruits are dipped in biomass according method from Alloue-Boraud *et al.* (2015). Fruits surface were disinfected by dipping ethanol 70% for 3 min, rinsed three times with sterile distilled water. After fruits are storage at 25° C during 14 days.

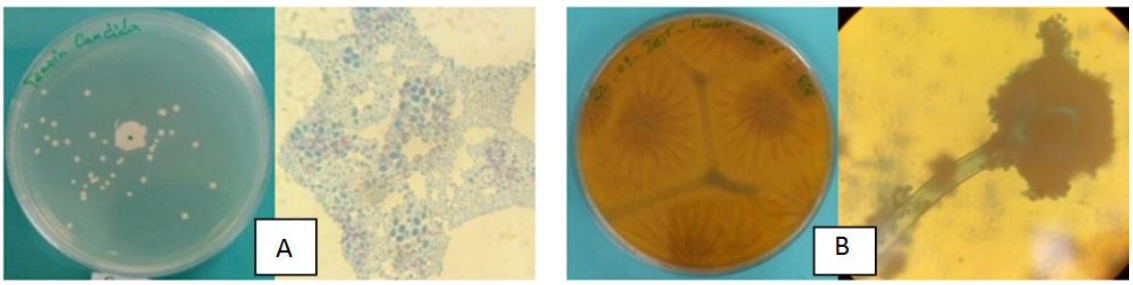
Results

Isolated and identified fungi on pineapple

Fusarium sp., *Aspergillus* sp. (a) and *Rhizopus* sp. were isolated on pineapples diseased while the yeast of the genus *Candida* and the fungi of the genus, *Aspergillus* (b) have been isolated from healthy pineapple. Isolated on healthy pineapple are shown in Fig. 1 and those isolated pineapple diseased in Fig. 2.

Pathogenicity test

After five days of artificial inoculation pineapples with the previously isolated fungi namely *Aspergillus* sp. (a), *Aspergillus* sp. (b), *Fusarium* sp. *Rhizopus* sp. and *Candida* sp., pineapples showed alterations that resulted in soft rottenness even reaching the heart of the pineapple and disagreeable odours (Fig. 3).



Candida sp. *Aspergillus* sp. (a) *Aspergillus* sp. (a)

Fig. 1. Macroscopic and microscopic view of *fungi* isolated on healthy pineapple (*Candida* sp. (A) *Aspergillus* sp. (a) (B).

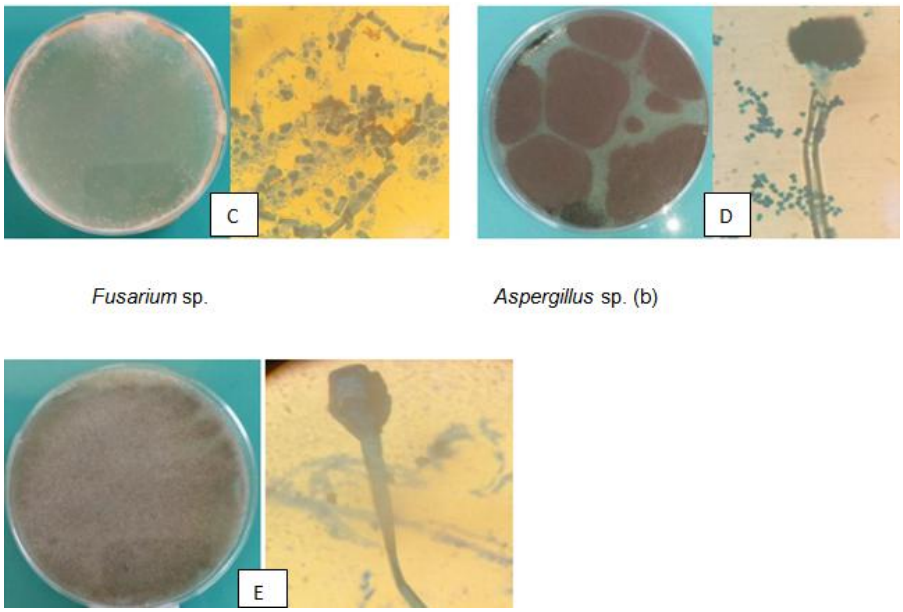


Fig. 2. Macroscopic and microscopic view of *fungi* isolated on diseased pineapple. *Fusarium* sp. (C) *Aspergillus* sp. (b) (D) *Rhizopus* sp. (E).

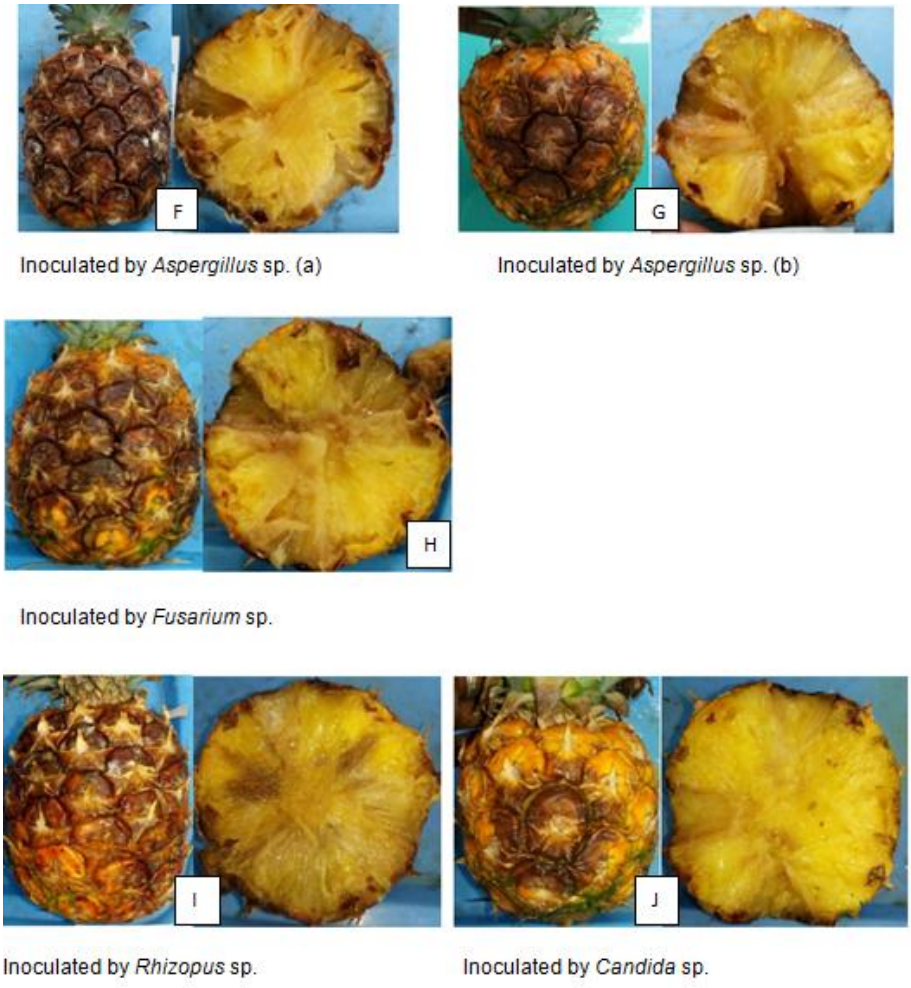


Fig. 3. Pathogenycity after 48 hours inoculation with fungi isolated. Inoculated by *Aspergillus* sp.(a) (F), Inoculated by *Aspergillus* sp.(b) (G), Inoculated by *Fusarium* sp (H), Inoculated by *Rhizopus* sp (I), Inoculated by *Candida* sp. (J).

Antagonism test in vitro

Inhibitions rates calculated for various confrontations (Microorganisms isolated and *B. subtilis*) are shown in table 1.

Table 1. Inhibition rates of *Bacillus subtilis* GA1 versus fungal isolated.

Fungi isolated	Inhibition Rates of *B. subtilis* GA1 (%)
Aspergillus sp. (a)	81.42± 0.34
Aspergillus sp. (b)	69 ± 0.29
Rhizopus sp.	64 ± 0.27
Candida sp.	60 ± 0.37
Fusarium sp.	57.14 ± 0.35

In vivo antagonist test

Bacillus subtilis GA1 reduced the incidence of microorganisms in the alteration of the pineapple through reduced spoilage diameter (Fig.4). *B. subtilis* GA1 was more effective on *Aspergillus* sp. (a).

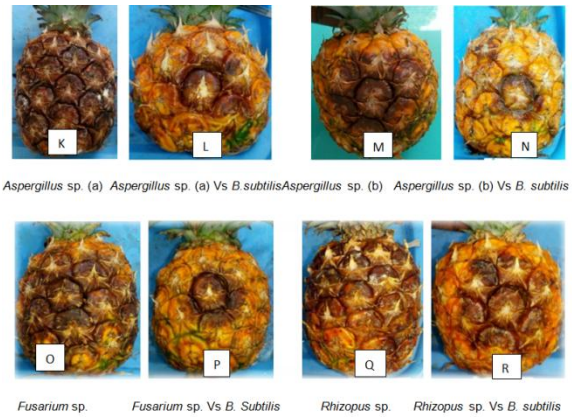


Fig. 4. *In vivo* inhibition of fungi by *Bacillus subtilis* GA1action on pineapple after incubation time. *Aspergillus* sp. (a) (K), *Aspergillus* sp. (a) Vs *B.subtilis* (L), *Aspergillus* sp. (b) (M), *Aspergillus* sp. (b) Vs *B. subtilis*(N), *Fusarium* sp (O) *Fusarium* sp. Vs *B. subtilis* (P), *Rhizopus* sp. (Q), *Rhizopus* sp. Vs *B. Subtilis* (R).

Test of fruit protection

Pineapple were immersed in the *B. subtilis* GA1 biomass and stored at the laboratory temperature (25 ± 0.1) did not show signs of deterioration after one week. Signs of deterioration appeared fourteen days after treatment with black spots on the fruit. However, within its pineapple showed an absence of yeasts and fungi, as well as the color of the heart of the pineapple (Fig5), the smell and the shape of the fruit that have been stored. Pineapples unprotected by *B. subtilis* GA1 showed signs of weathering at seven days of storage, with the presence of fungi inside the fruit (Fig.6).

Fig. 5. Protection with biomass from *Bacillus subtilis* GA1.

Fig. 6. No protected fruits of pineapple.

Discussion

Pineapples fruits are very vulnerable to microbial contamination products from the picking up conservation. Indeed, according to FAO (1995), microorganisms such as fungi can infected and caused spoilage pineapple if picking transportation and conservation conditions are poorly implemented. Pineapples fruits are affected by variety of microorganisms such as pathogenic fungi which cause their degradation, resulting in the change of taste, smell, appearance or texture, resulting in less attractive and poisonous fruits (Barth *et al*., 2009). These fungal activities can also lead to mycotoxin contamination, and could pose a risk to consumer health (Koffi-Nevry *et al.,* 2011). Fungi identified in pineapple diseased were represented by *Aspergillus* sp. (b), *Fusarium* sp. and *Rhizopus* sp. While *Aspergillus* sp. (a) and Candida sp. for healthy pineapple variety MD-2. These results are similar with the work of Ewekeye *et al.* (2013), who have shown that fungi commonly involved in the alteration

of most of fruits and vegetables are *Aspergillus* sp., *Fusarium* sp. and *Rhizopus* sp. This is in agreement with the report of Jolaosho *et al.* (2010) who isolated *Rhizopus* sp. of pineapple slices packed in Ogun State. Onuorah *et al.* (2013), have confirmed the presence of *Aspergillus* sp. and *Candida* sp. in healthy pineapple in Anambra State, Nigeria. Akinmusire (2011) also isolated *Aspergillus* sp. and *Candida* sp. from pineappleamples in Maiduguri, north east of Nigeria. Effiuvwevwere and Oyelade (2000) even reported in their study that *Aspergillus* sp. and *Candida* sp. are responsible for rottening, a disease of the fruit of the pineapple. According to Onuorah *et al.* (2013), the presence of fungi in pineapple fruits is a risk for consumer's health. The presence of fungi in pineapples can be also linked to a number of factors such as poor handling and poor processing, use of contaminated water during washing, cross contamination, use of dirty utensils such as treatment knives and plates, physiological and physical conditions of production, and extrinsic parameters to which they are subject. The results of pathogenicity tests on pineapples fruits show that all fungi isolated were responsible for the deterioration of pineapple fruit and can also alter various fruits other than their original host. The tests also demonstrated the link between changes in fruits and presence Opening in pineapple. Indeed the fungi causing the fruit changes when they enter the pineapple by mechanical injuries such as bruises and wounds as highlighted Zitter (1985). They can also enter the fruit by damages caused by any kind of pests. This damage is to be feared for fruit undergoing storage (DAF, 2006). These alterations could be explained by the ability of these fungi to metabolize sugars and nutrients in the pineapple and also to grow in very low pH conditions with the pineapple high water content. Here *Aspergillus* sp. pathogenicity results and *Candida* sp. isolated from pineapple are similar to those of Efiuvwevwere (2000) who reported that *Aspergillus* sp. and *Candida* sp. are responsible for rotting pineapples. The *B. subtilis* strain reference GA1 has shown its ability to inhibit mushrooms such as *Aspergillus* sp. (a), *Aspergillus* sp. (b), *Rhizopus* sp., *Candida* sp. and *Fusarium* sp.isolated from pineapple fruits. These results are similar to those obtained by Alloue-Boraud *et al.* (2015). They have found that rates of *B. subtilis* GA1 inhibitions were between 59.37% and 84.78% on pathogens strains duringmangoes conservation. This reduction in the incidence of microorganisms in the alteration of the pineapple can be explained, by the property of the reference strain which is a rapid and extensive colonization injury sites. This presence would hinder the establishment of the fungal pathogen in wound sites by a reduction space and available nutrients. Droby *et al.* (1989) and Zhao *et al.* (2008) reported that application of the antagonist in the wound sites before infection pathogen is necessary to ensure better colonization and a maximum rate of protection by reducing the incidence of pathogen infection, and secondly for the production by *B. subtilis* GA1 lipopeptide namely, fengycin which is recognized for its strong antifungal activity.

The test on protection fruit revealed that *B. subtilis* GA1 has an inhibitory activity on pathogens. Indeed, colonization of the pineapple by *B. subtilis* GA1 has able to prevent the growth of pathogenic fungi in fruit. This which has kept the firmness as well as its characteristics organoleptic. These results of this study are consistent with those of the work of Alloue-Boraud *et al.* (2015) who worked on mango. However, signs of alterations observed on fruits after two weeks could be related to pathogenic fungi that familiar with the substrate environment and constitute the natural flora of pineapple. They adapt more easily to product unlike *B. subtilis* that needs time to adapt. Inhibition of pineapple alteration flora by *B. subtilis* come from its ability to produce lipopeptides having antibacterial and antifungal by the bursting of the wall cell fungi (Ongena, 2014). A key feature of *B.subtilis* lipopeptides lies in their surface active properties can be explained by the decrease in surface tension of the change and disruption of bilayer lipid (Deleu *et al.,* 2003; Heerklotz and Seelig, 2007). The obtained protection level is encouraging for the development of a biological control method.

Conclusion

The results presented here demonstrate that *B. subtilis* strain inoculated on pineapple pulp can readily germinate allowing significant cell populations to establish and efficient in vivo synthesis of lipopeptides which could be related to disease reduction.

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