Ban Koffi et al., *Cogent Food & Agriculture* (2017), 3: 1394249
https://doi.org/10.1080/23311932.2017.1394249

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FOOD SCIENCE & TECHNOLOGY | RESEARCH ARTICLE

Enhancement of mango fruit preservation by using antimicrobial properties of *Bacillus subtilis* GA1

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Received: 28 March 2017
Accepted: 09 October 2017
First Published: 23 October 2017

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Reviewing editor:
Fatih Yildiz, Middle East Technical University, Turkey

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Abstract: Pesticides are used to eradicate negative impacts of pests on food crops. Fungi are main microorganisms that affect quality. Some bacteria, *Pseudomonas* F19, *Bacillus subtilis* GA1 have been studied for their biotechnological and phytopathogenic properties. The aim of this work is to study in phenotypic way the microorganisms associated to healthy and altered mango fruits and find out *B. subtilis* GA1 aptitude for inhibition of growth of those spoil mango. *B. subtilis* GA1, recognized for its properties was tested directly *in vitro* and *in vivo*. *Colletotrichum* sp. and *Aspergillus* sp. were naturally associated to mango. *Colletotrichum* caused greatest alteration with lesion length of 54.2 ± 1.92 mm after three days. *In vitro* inhibitory activity test of strain GA1 showed a strong inhibition of *Colletotrichum* at 72.78 ± 2.90%. In same time, test of *in vivo* inhibitory activity has reduced mango spoilage at 89.29 ± 3.84%.

Subjects: Environment & Agriculture; Food Science & Technology; Engineering & Technology

Keywords: mango; *Bacillus*; *Colletotrichum*; *Aspergillus*; *Candida*; preservation

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PUBLIC INTEREST STATEMENT

Because of their potential source of nutrients, fruits consumption is recommended for people well being. For very long time, preservation of agricultural products and mainly food crops against pests has been made through the use of chemical substances. However, it has been demonstrated that these products are sources of environmental pollution and public health problems. They are responsible for acute or chronic contaminations at the consumers' level. Because of harmful effects of pesticides, consumers are demanding food products with good sanitary qualities. In the same time, it was found that certain microorganisms such as *Pseudomonas* and *Bacillus* produce metabolites which are safe for consumer's health and the environment. These microorganisms are used as biopesticides to prevent fruits alteration.

This study aims to highlight the ability of certain microorganisms to preserve mango fruits against alteration due to microorganisms such as *Colletotrichum* sp., *Aspergillus* sp. and *Candida* sp.

1. Introduction

Mango (*Mangifera indica* L.) is considered as the most popular fruits usually eaten by millions of people in the tropical area especially in the developed countries. The annual production of this fruit is more than 100,000 tons in Côte d'Ivoire. Most of this production is exported (Kouassi, 2012). The most produced mango varieties are Kent, Keitt and they offer logistical advantages which make them the third largest supplier markets (Gerbaud, 2008; Moulin, 2007). However, the exported fruits are damaged by several fungi such as *Colletotrichum gloeosporioides* responsible of the anthracnose, the major constraint of production and exportation of fresh mangoes fruits in Côte d'Ivoire (Akem, 2006). This disease is considered as the most devastating of mango fruits. Anthracnose deteriorates fruits quality and also causes severe post harvest losses (Lakshmi, Reddy, & Pascal, 2011). The fruit can be infected in the field through lenticels or wounds during harvest, transportation or storage activities. The symptoms of Anthracnose on green infected fruits remain latent and invisible. The symptoms are most conspicuous and important on ripened or overipened fruits (Silva & Michereff, 2013). Infected mangoes offer rounded brown to black lesions with an indefinite border on their skin. Different lesion sizes can coalesce and cover extensive area of the fruit skin. In a case of severe infection, the fungi can invade the pulp and reach the stone. These necroses affect the commercial value of the fruits and induce income losses at the producers level and all the actors involved in the mango chain value.

Application of synthetic fungicides is the major approach for the control of fruits diseases. It has been reported that hot benomyl dips controls anthracnose on harvested mango fruits (Kim, Brecht, & Talcott, 2007). Also, using fungicides at high concentrations over a long time can result in pathogen resistance (Tian, Liu, Zhang, & Meng, 2010). The utilisation of chemical pesticides can also be source of public health problems. Additionally to that, public concern about fungicide residues. This brinks researchers to find new technologies for fitting against fruits diseases. Some of Gram positive bacteria, such as *Bacillus subtilis* GA1, are able to stop inhibition growth of spoilage microorganisms responsible of fruits quality deterioration. They are able to produce under certain conditions, antibacterial or antifungal metabolites that reduce growth and development of undesirable microorganisms.

The main objective of this study was to find out *B. subtilis* GA1 aptitude to stop or reduce growth and development of microorganisms responsible of mango fruits alteration in order to enhance mango fruit conservation. So, microorganisms that naturally associated to altered or inaltered mango fruits are isolated and *B. subtilis* GA1 biological activity are tested on them.

2. Materials and methods

2.1. Biological materials

One hundred and three samples of altered or unaltered mango fruits, Keith variety, were collected from fruit markets of Abidjan Plateau, Cote d'Ivoire. A purified and well identified *B. subtilis* strain GA1, from the collection of Walloon Center of Industrial Biology (CWBI), University of Liege Gembloux Agrobio Tech (Belgium) was used for different tests.

Isolates of microorganisms got from healthy and altered mango fruits, Keith variety were used for the different tests. Mangoes were obtained from a commercial warehouse in Abidjan-Plateau, Côte d'Ivoire.

2.2. Pathogen isolation

One hundred and three fruits were washed with sterilized water before removing fragments of fruit from margins of decayed and healthy tissues with a sterile scalpel (Djossou et al., 2011). The fragments were placed on Petri dishes containing PDA culture medium (Becton, Dickson and Company, Le pont de Claix, France) supplemented with chloramphenicol in order to prevent bacterial development. The Petri dishes were incubated at 27°C during 5to 7 days. Isolates of microorganisms were

identified by macroscopic and microscopic examination based on identification keys (Botton et al., 1990; Guiraud, 1998; Mathur & Kongstal, 2003).

2.3. Microbial inocula preparation for in vitro and in vivo assays

Bacterial endospore suspensions used in biocontrol experiments were prepared from 72 h old cultures of *B. subtilis* GA1 grown at 30°C in agitated flasks (105 rpm) in 500 ml of 863 media described by Jacques et al. (1999). Cultures were centrifuged at 35,000 rpm for 20 min and the biomass pellet was washed three times in physiological water (9‰). The supernatant was collected in sterile tube and was filtered with a sterile membrane Millipore of 0.2 µm. Fungi concentration was determined by plate count on PDA medium. They were then resuspended in a sterile distilled water to obtain the final desired concentration of 10^5 CFU/ml.

2.4. Pathogenicity assay

Twenty-five healthy fruits without lesion had their surfaces disinfected by immersion during 5 min in 1% sodium hypochlorite solution.

They were rinsed three times with sterile distilled water. Wells measuring 3 mm wide and 5 mm deep were created with a sterile cork borer (Regnier, du Plooy, Combrinck, & Botha, 2008). Fruits were treated with fungi isolated (yeast or mould) by adding 50 µl of cell containing 10^5 CFU/ml in the wells. Control sample were inoculated with 50 µl of sterile distilled water.

Inoculated fruits were stored at 25°C room temperature during 5 days. The fruits that develop microorganisms are recovered and microorganisms are identified as described previously.

Each treatment was achieved 5 times. The lesion diameter (LD) were measured by using a ruler.

2.5. In vitro antagonism activity developed by B. subtilis GA1

*B. subtilis*GA1 was tested for its ability to inhibit the growth of various fungi plant pathogens in Petri dishes containing PDA medium. Two sterile disks of 6 mm has been laid to 3 cm of Petri dishes center. One dish carried out *B. subtilis* GA1 and the other one carried out the fungi pathogens. A second Petri dish contained only the fungi pathogens. 2 µl of microbial suspension were dropped on every sterile disk. The radii of fungal growth in both the control and dual culture plates were measured 3 days after incubation. The rate inhibition was defined as the difference between the distance of the growth in the dual culture plate (r in centimeters) and the fungal growth radius (R in centimeters) of the control plate, where $\Delta R = R - r$ and the percentage inhibition of fungal growth was calculated using the following equation (Erdogan & Benlioglu, 2010).

$$\% \text{ Inhibition} = \left(\frac{R - r}{R}\right) \times 100$$

2.6. In vivo antagonism activity developed by B. subtilis GA1

Sixty-four healthy and freshly harvested mature mango fruits were carefully selected on the basis of absence of any disease or wounding symptoms. The fruits were washed with soapy water and rinsed 3 times with sterile distilled water. Then, fruits were surface sterilized by swabbing with 70% ethanol solution. 6 mm wide and 3 mm deep wells were then artificially created with a sterile cork borer. Fruits were treated with *B. subtilis* GA1, the antagonist bacteria by adding 50 µl of cell (10^5 CFU/ml) or 25 µl of its supernatant 24 h prior to pathogen challenge. Infection with fungal pathogens were realized in all cases 24 h later, by adding the same volume of a microbial suspension prepared as described above in order to introduce 10^5 (10^5 CFU/ml) conidia per site (Toure, Ongena, Jacques, Guiro, & Thonart, 2004). Two other treatments were also carried out. The first one is a control test regarding fruits that were only inoculated with 50 µl of sterile distilled water and the second one is a control disease test based on a none treated fruits with the antagonist bacteria but inoculated with the pathogens. Disease incidence was evaluated 3–7 days after pathogen challenge. It based on the diameter of spreading lesions developed around infected sites. Results were expressed as percentage disease reduction by using the following relation:

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$$P = (D_t - D_e)/(D_t - K)$$

where K is the wound diameter (6 mm) and D_t and D_e are lesion diameters measured on disease controls and on treated fruits, respectively (Toure et al., 2004).

2.7. Statistical assay

The software R version i386 3.2.2 for Windows was used for statistical analyses. The homogeneity of variances was tested by ANOVA and data from experiments with the same set-up were pooled for analysis when interaction between experiment and treatment was not significant at $p = 05$. Means from the different treatments were compared by the method of Tukey (least significant difference at $\alpha = 0.05$).

3. Results

3.1. Isolated fungi from tested mango fruits

Colletotrichum sp., *Aspergillus* sp. and *Candida* sp. were the main pathogens isolated from mango fruits.

Colletotrichum sp. was the most important pathogen isolated both healthy and spoiled fruits at 100% level. *Aspergillus* sp. and *Candida* sp. are also associated to healthy and spoiled fruits at a respective level of 70 and 30 (%). All these three fungi are involved in mango fruits deterioration (Table 1).

3.2. Pathogenicity assay

Pathogenicity tests carried out on mango fruits with each of the isolated fungi reveal fruits alteration after 3 days (Figure 1).

Impact of the pathogens on the fruits quality is shown in Table 2. The most important alteration was observed on fruits artificially inoculated with *Colletotrichum* sp. The diameter of the lesion was estimated at 54.2 ± 1.92^{b} mm. However, the alteration length of fruits inonculated with a combination of *Aspergillus* sp. and *Colletotrichum* sp. was estimated at 55.6 ± 3.50^{b} mm and the one with the total pathogens was estimated at 61 ± 3.16^{d} mm. Mango artificially inoculated with *Candida* sp., is almost alterated (Figure 1(a)). Alteration from *Aspergillus* sp. was blackened (Figure 1(b)). The alteration of fruits inoculated with *Candida* sp. and *Aspergillus* sp. started 3 days after artificial inoculation and the alteration of those inoculated with *Colletotrichum* sp. showed signs of alteration from the 2nd day with a total alteration 5 days later (Figure 1(c)). The lesion alteration diameters (Table 2) are different according to microorganisms with $p < 0.05$. Fruits spoilage was important with *Colletotrichum* sp. with a lesion diameter of 54.2 ± 1.92 mm, followed by *Aspergillus* sp. with 38 ± 1.58 mm of lesion diameter. The greatest lesion diameter of 61 ± 3.16 mm was

Table 1. Frequency percentage of various fungal from samples

Fungal pathogens	Positives samples Percentages (%)		
	Spoilage mango	Healthy mango	Total
Candida sp.	60	40	30
Aspergillus sp.	80	60	70
Colletotrichum sp.	100	100	100

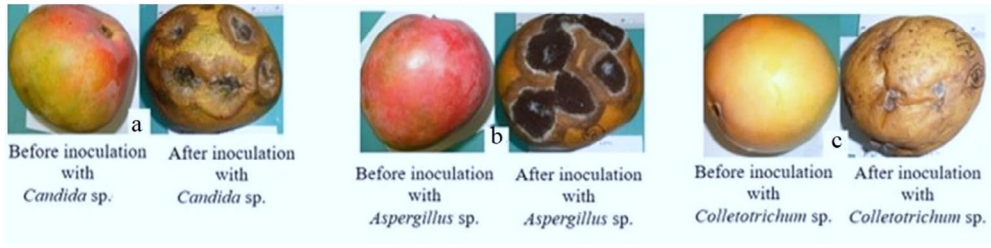

Figure 1. Effect of pathogens fungal isolated on mongo.

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Table 2. Lesion diameters (mm) induced by fungi isolated after three treatments days

Fungal pathogens	Lesions diameters (mm)
Candida sp.	26.8 ± 1.79[a]
Aspergillus sp.	38 ± 1.58[c]
Colletotrichum sp.	54.2 ± 1.92[b]
Aspergillus sp. and *Colletotrichum* sp.	55.6 ± 3.50[b]
All fungi isolated	61 ± 3.16[d]

N.B: a, c, d values with different letters are significantly different at $p < 0.05$.

obtained when the inoculation was carried out with the combination of all identified fungi. It is following by inoculation with combination of *Colletotrichum* sp. and *Aspergillus* sp. with a lesion diameter of 55.6 ± 3.50 mm (Figure 2).

3.3. Assessment of inhibition of bacterial growth

Ability of Strain GA1 inhibition or reduce the growth of *Colletotrichum* sp., *Aspergillus* sp. and *Candida* sp. is shown in Table 3. The inhibition level runs from 71.50 ± 1.66 to 75.79 ± 1.32% with an intermediate value estimated at 74.54 ± 0.94% respectively for *Candida* sp., *Aspergillus* sp. and *Colletotrichum* sp.

In vivo treatments of fruits treated with strain GA1 cell were not alterated 5 days after pathogen inoculation (Figure 3).

Inhibition of Colletotrichum sp., *Candida* sp. and *Aspergillus* sp. is observed respectively at level of 92.48 ± 1.74%, 90.45 ± 2.57 and of 84.60 ± 1.80% after testing with *Bacillus subtilus* GA1. After 10 days, treated fruits with strain GA1 cells showed a rate mean inhibition value of 89.29 ± 3.84% for

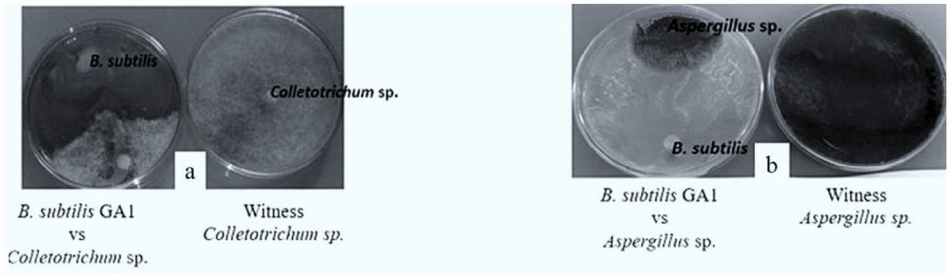

Figure 2. *In vitro* growth inhibition of *Colletotrichum* sp. (a) and *Aspergillus* sp. (b) by *Bacillus subtilis* GA1 on Potato Dextrose Agar (PDA) medium.

Table 3. Inhibition (%) of *Candida* sp., *Aspergillus* sp. and *Colletotrichum* sp. after tested against *Bacillus subtilis* GA1

Fungal pathogens	*Candida* sp.	*Aspergillus* sp.	*Colletotrichum* sp.
Mycelium growth inhibition (%) by *B. subtilis* GA1	71.50 ± 1.66[a]	75,79 ± 1.32[b]	74.54± 0.94[b]

Note: Values with b different letters are significantly different for $p < 0.05$.

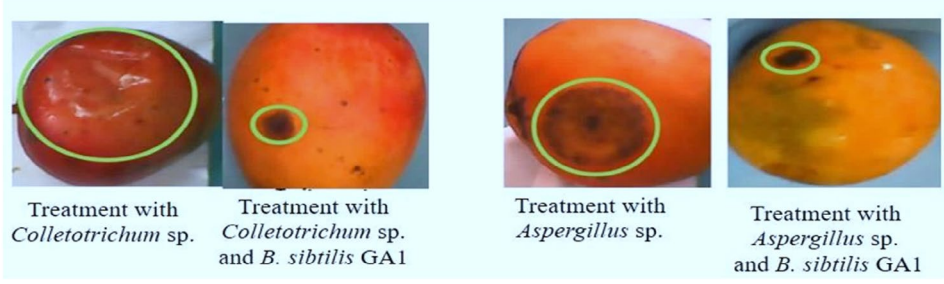

Figure 3. Examples of fruits observed 5 days after treatment with *Bacillus subtilis* GA1 against *Colletotrichum* sp. And *Aspergillus* sp.

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Table 4. Effect of *B. subtilis* GA1 treatment on growth of *Colletotrichum* sp., *Candida* sp. and *Aspergillus* sp.

Treatments	Fungal pathogens		
	***Colletotrichum* sp.**	***Candida* sp.**	***Aspergillus* sp.**
B. subtilis GA1 Cell (%)	92.48 ± 1.74[a]	90.45 ± 2.57[b]	84.60 ± 1.80[c]
B. subtilis GA1 supernatant (%)	90.31 ± 2.31[a]	87.19 ± 5.26[b]	85.38 ± 3.38[c]

all treated mangoes. However, the maximum rate reduction was obtained with *Colletotrichum* sp. at a level of 90.45 ± 2.57% and the lowest one was obtained with *Aspergillus* sp. at a level of 85.38 ± 3.38. A mean reduction of 87.84 ± 4.09% was maintained during 10 days. There is no difference between percentages alteration reduction (Table 4) for all treatments with $p < 0.05$.

4. Discussion

This study revealed that *Colletotrichum* sp., *Apergillus* sp., *Candida* sp. were the main pathogens isolated from mango fruits particularly Keith variety collected in Abidjan, Côte d'Ivoire. This result is in accordance with those obtained by Alloue-Boraud, Koffi, Dadie, Dje, and Ongena (2015) who isolated *Candida* sp. and *Colletotrichum* sp. from Kent, a variety of mangoes cultivated in Cote d'Ivoire. Others fungi such as *Colletotrichum gloeosporioïdes*, *Apergillus flavus* and *Apergillus niger* were also isolated from mango fruits in Nigeria. *Aspergillus* sp. found on mangos could be source of mycotoxins, such as ochratoxins in the fruit (PIP, 2013). Primary habitat of *Candida* sp. is digestive tract of men and warm blooded animal. It could be distributed surrounding soil, plants, foods and forages. It's presence on fruits are lack of hygiene according to Rispail (2008) and N'guettia, Kouassi, and Kouakou (2014). Post-harvest mango infection could be also influenced by orchards, market sanitation and transportation (N'guettia et al., 2014). That might favored the accumulation of inoculums from one crop to another during the market cycle. This contribute to enhance the rate infection.

The pathogenicity of isolated germs was confirmed by the deterioration of fruits quality and necrosis observed on fruits skin. That observation is in agreement with Onyeani, Osunlaja, Owuru, and Sosanya (2012) who have proved that these microorganisms are responsible of mangos infection in Asia. The degree of pathogenicity and virulence varies from one organism to another. This result corroborate with that of Kouame et al. (2011). Also, Onyeani and Oguntade (2015) demonstrated the ability of *C. gloeosporioides* to necrosis strawberry fruits inoculated with it. Strain GA1 aptitude to protect mango fruits against *Colletotrichum* sp., *Apergillus* sp. and *Candida* sp. spoilage is confirmed by those of Toure et al. (2004) and N'guettia et al. (2014). In fact, fruits treated with vegetative cells or supernatant from strain GA1 allowed an effective control of the pathogens. Supernatant from strain GA1 also provided a strong protective effect that was similar to the one observed with live cells. This suggested the production of metabolites by strain GA1 that are involved in the growth inhibition of pathogens. One of these metabolites could be the lipopeptides which were studied by Toure et al. (2004) on apple. Study achieved by Ongena (2014) showed also the antifungal properties of these lipopetides.

5. Conclusion

It has been demonstrated that *Colletotrichum* sp., *Aspergillus* sp., *Candida* sp. are phytopathogenic microorganisms naturally associated to healthy mango fruits and are involved in mango fruits alteration. However, vegetative cells and supernatant obtained from biopesticides such as *B. subtilis* GA1 could contribute to prevent agricultural products and environment against alteration. They could be efficient in the biocontrols of these phytopathogenic microorganisms.

Research of wide spectre of bacteria susceptible to be used as biopesticides should be carried out in order to reduce the use of pesticides and their negatives impacts on the environment, food crops quality and on public health.

Acknowledgement
The authors gratefully acknowledge both Microbiology Unit of the Technological Research Station of the National Agricultural Research Center of Côte d'ivoire, the Unit of Bioindustry of the University of liege, Gembloux Agrobiotech (Belgium for respectively their financial, technical support and technical assistance.

Funding
The authors received no direct funding for this research.

Competing Interests
The authors declare no competing interest.

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Citation information
Cite this article as: Enhancement of mango fruit preservation by using antimicrobial properties of Bacillus subtilis GA1, Louis Ban Koffi, W.A. Mireille Alloue-Boraud, Adjéhi Thomas Dadie, Saman Hervé Koua & Marc Ongena, *Cogent Food & Agriculture* (2017), 3: 1394249.

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Cogent Food & Agriculture **(ISSN: 2331-1932) is published by Cogent OA, part of Taylor & Francis Group.**