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Reduction of fungal spoilage in *Amélie* variety mango (*Mangifera indica* L.) using biocontrol agents

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Abstract

The mango is today one of the five most produced and consumed fruits in the world after the orange, the banana, the grape and the apple. It is one of the three most exported fruits from Côte d'Ivoire with banana and pineapple. However, the mango sector faces enormous post-harvest losses due to diseases and handling activities. It is in this context that the objective of this work is to reduce post-harvest losses of the *Amélie* variety mango, using formulations based on biocontrol agents (*Pseudomonas fluorescens* and *Bacillus subtilis*) against fungal germs spoilage. To do this, isolation and identification of spoilage fungal strains on 50 spoiled *Amélie* variety mangoes were carried out. Two liquid formulations based on the two biocontrol agents were produced and tested on the isolated fungal strains. The results of this study showed that *Colletotrichum* and *Lasiodiplodia* were identified as genera responsible for mango spoilage. *In vitro* antagonist tests carried out with the formulations inhibited the growth of *Colletotrichum* and *Lasiodiplodia*. The inhibition rates of the *Bacillus subtilis* formulation were between 76.63% and 89.06% and those of *Pseudomonas fluorescens* were 78.21% to 83.57%. For the consortium (*Bacillus subtilis* + *Pseudomonas fluorescens*), the inhibition rates of the formulations were between 87.73% and 88.97%. Therefore, *Bacillus subtilis* and *Pseudomonas fluorescens* could be used as biocontrol agents in the fight against fungal spoilage of mango in Côte d'Ivoire.

Keywords: Mango; Fungal spoilage; Antagonistic activity; formulation; *Bacillus subtilis*; *Pseudomonas fluorescens*

1. Introduction

Fruits have always been part of the daily human diet. Having very attractive colors, tastes and aromas, they constitute an inexhaustible source of nutrients. They are often considered "functional foods" thanks to the rich content of various micronutrients such as phenolic compounds, minerals, vitamins. Among these fruits, the mango is one of the five most cultivated and appreciated fruits in the world, after the orange, the banana, the grape and the apple [1]. In the 1980s, mangoes and the Amélie variety constituted 90% of mango orchards in West Africa, today we observe a domination of Floridian varieties such as *Kent* and *Keitt*, and to a lesser degree, the *Amélie* [2]. At the regional level, mango production promotes significant economic benefits through the creation of tens of thousands of jobs from the orchard to the packing station [3]. Côte d'Ivoire represents the first African country and the third country globally, supplier to the European market after Brazil and Peru. Mango exports generate more than 7 billion FCFA in revenue. They provide producers with around 1 billion FCFA [4]. The mango represents the third fruit exported by Côte d'Ivoire after pineapple and

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banana [4]. The annual production of Côte d'Ivoire is estimated at more than 100,000 tonnes [5]. Despite its economic and nutritional importance, the Ivorian mango sector faces several problems of post-harvest spoilage due to the increase in pathogens [6]. Among the diseases that affect mango production, peduncle rot has become increasingly important. This rot is attributed to *Lasiodiplodia theobromae* [7]. Anthracnose, caused by *Colletotrichum gloeosporioïdes*, represents the second major constraint linked to the production and conservation of mangoes [11]. In fact, tens of tons of mangoes rot in orchards every year. This situation leads to enormous losses of earnings at the producer level. Post-harvest losses are in fact estimated at around 30 to 35% of total production for the equivalent of 3.3 billion FCFA/year [4]. The control of diseases affecting fruits, and more specifically mangoes, is mainly based on the use of fungicides before harvest or just after harvest. Currently, the fight against pests is mainly based on the use of synthetic pesticides [9]. Indeed, the chemical control methods used for disease reduction constitute one of the constraints of the increasing resistance of pathogens and the maximum limits of pesticide residues in fruits imposed by importing countries [10]. The disadvantages of chemical pesticides have led to the search for alternatives for the development of other products more respectful of the environment and the health of the consumer in order to fight against phytopathogens. Thus, biological control through the use of bacteria generally recognized as safe (GRAS) has been widely studied. *Bacillus subtilis* and *Pseudomonas fluorescens*, existing biological control agents, have been used for a long time as biopesticides due to their antibacterial and antifungal properties. Thus, several research studies have demonstrated the effectiveness of *Bacillus subtilis* and *Pseudomonas fluorescens* in the biological control of fruit pathogens [11,12]. However, there is little data on the formulation of bacterial biopesticides in the biological control of fungal diseases of mango in Côte d'Ivoire. It is in this context that this study takes place, the objective of which is to inhibit post-harvest fungal germs of the *Amélie* variety mango using the formulation of two biocontrol agents (*Pseudomonas fluorescens* and *Bacillus subtilis*).

2. Material and methods

The plant material used for this study consisted of spoilaged mangoes of the *Amélie* variety (Figure 1), collected from the orchards of Ferkessédougou (Côte d'Ivoire). The biocontrol agents used are *Bacillus subtilis* and *Pseudomonas fluorescens*. These strains come from the collection of the Laboratory of Biotechnology and Food Microbiology of Nangui ABROGOUA University (Abidjan, Côte d'Ivoire). They were isolated from the rhizosphere of the mango tree and stored in cryovials in a freezer at -80°C.

Figure 1 Spoiled *Amélie* variety mango

2.1. Sampling

Five (05) samples each comprising ten (10) spoiled mangoes at the senescence stage, i.e. 50 spoiled mangoes, were taken directly from five (05) plots of *Amélie* variety mangoes at the rate of one plot per producer. Once collected, the different fruits were packaged separately in plastic stomacher bags, labeled, sealed then kept in a cooler containing dry ice where the temperature was maintained at 4 °C.

2.2. Isolation of mango spoilage fungal strains

The direct contact isolation technique on PDA agar as described by [13] was used to isolate mango spoilage fungi. Three spoiled mangoes were chosen at random from each sample then washed with tap water and disinfected with 2% bleach for 2 min in order to eliminate exogenous microflora. The mangoes were rinsed twice with sterile distilled water to remove bleach residue. The mangoes were dried in plastic bins on household paper spread in the plastic bins. The plastic bins were first washed with a sponge and soap and rinsed thoroughly with tap water then disinfected with 2% bleach soaked on household paper. After the mangoes dried, they were disinfected with household paper soaked in 70% ethanol before sampling. The mango skin and spoiled mango flesh were individually cut into small pieces of

approximately 1 centimeter in diameter using a sterile scalpel. Subsequently, three fragments of mango skins and mango flesh previously cut, were taken using sterile forceps then the fragments of mango skins were placed on either side on a Petri dish containing Potato Dextrose Agar (PDA). Similarly, three fragments of mango flesh were placed on either side of a Petri dish containing Potato Dextrose Agar (PDA). These fragments of mango skins and mango flesh were placed on the petri dishes in triplicate each. Isolation was carried out for all mangoes selected from all mango samples. Plates were incubated at 28 °C for 5–7 days. To obtain a pure strain, several subcultures on PDA medium were carried out. The choice of species to be sampled was made macroscopically, taking into account the color and appearance of the colonies. Once these are isolated in pure culture, they are subcultured on PDA medium to measure their apical growth speed and observe the macroscopic and microscopic characteristics.

2.3. Identification of fungi isolated from mango

The macroscopic identification of isolated fungi was carried out according to the method used by [12] through an examination of the culture on PDA agar. The cultural characteristics determined were the appearance of the colonies (fluffy, woolly, cottony, velvety, powdery or granular), the relief of the colonies (flat, convex, pleated, etc.), the color of the colonies (white, cream or colored, yellow, orange, brown, green, gray up to black), the size of the colonies (small, extensive or invasive), the speed of growth (fast or slow) as well as the color of the back of the Petri dish and the diffusion of the mycelium in agar. The method described by [14] was used for the microscopic identification of molds. To do this, a drop of methylene blue was placed in the center of a slide. Subsequently, a filament of mold was collected using with sterile forceps then placed in the methylene blue in the center of the object slide. The object slide was covered by a coverslip. The observation was made with a LEICA DM750 optical microscope with an X40 objective. The characteristics observed were the appearance of the mycelium (compartmentalized or not), the presence and shape of the spores (oval, spherical, round, etc.), the shape of the conidial heads and the size of the conidiospore (short or long). The frequency of mushroom isolation was calculated according to the method of [15].

$$\%IF = NI/TNI \times 100$$

- IF: Isolation frequency in percentage
- NI: Number of isolations of a fungal genus,
- TNI: Total number of fungal genera isolations.

2.4. *In vitro* antifungal activity of *Bacillus subtilis* and *Pseudomonas fluorescens*

The antagonism test was carried out with the aim of verifying the existence of an antifungal potential of *Bacillus subtilis* and *Pseudomonas fluorescens* on each of the isolated molds. The *in vitro* antifungal activity of *Bacillus subtilis* and *Pseudomonas fluorescens* bacterial strains was carried out with 24-hour cultures. Thus, this test was carried out according to the method described by [16]. The two bacterial strains, *Bacillus subtilis* and *Pseudomonas fluorescens*, were each spread on Petri dishes containing YPGA medium by making a longitudinal streak dividing the dish into two equal parts using a sterile platinum loop. Subsequently, a fragment of the mycelium of the same fungal was deposited on either side of the streak at a distance of 1 cm from the edge of the box. This operation was carried out with all fungal isolates. The witness boxes have were seeded in the center of the Petri dish only with the fungi where there was no seeding with *Bacillus subtilis* and *Pseudomonas fluorescens*. Incubation was carried out at 30 °C for 7 days. The evolution of the confrontation of the colonies of the fungi and the strains of *Bacillus subtilis* and *Pseudomonas fluorescens* were observed every day until the seventh day. After 7 days, the percentage of growth of the fungi in the boxes was determined using the method of [17], then the inhibition rate was deduced according to the following formula:

$$\text{Inhibition rate} = [(R - r) / R] \times 100$$

- r: The radial growth of the microorganism with antagonist,
- A: The radial growth of the microorganism without antagonist confrontation.

2.5. Formulation of bacterial biocontrol agents based on *Bacillus subtilis* and *Pseudomonas fluorescens*

2.5.1. Carrying out the pre-culture

The pre-culture was carried out by preparing 100 ml of YPG (Yeast extract-peptone-glucose) medium distributed in sterile Erlenmeyer flasks at a rate of 50 ml per flask. Then, these different media were inoculated from colonies of *Bacillus subtilis* and *Pseudomonas fluorescens* obtained on agar medium. The pre-culture was subsequently incubated at 30°C for 8 h with shaking at 155 rpm. This pre-culture was used to inoculate 200 ml of bacterial culture.

2.5.2. Preparation of bacterial culture

The bacterial culture was carried out by transferring 200 ml of Yeast extract-Peptone-Glucose (YPG) broth into two jars of 300 ml each. These media were seeded respectively with 50 ml of pre-culture of *Bacillus subtilis* and *Pseudomonas fluorescens*. They were then incubated at 30 °C with shaking at 155 rpm for 72 hours. The dissolved oxygen was set at 30% and the air flow rate was adjusted between 2 and 2.5 L. After 72 h, the resulting bacterial culture was centrifuged at 6000 rpm for 10 minutes using a refrigerated centrifuge (ACM- CFG-54251, India). The supernatants were collected in sterile 300 ml jars and kept at 4 °C. To these supernatants, formulation adjuvants such as glucose, glycerol, extra virgin olive oil and Tween 20 were added to obtain the bacterial biological product in liquid form [18].

2.5.3. Liquid formulation of biocontrol agents

The formulation was carried out according to the modified method of [19]. Thus, different liquid formulations were prepared by mixing 75 ml of bacterial supernatant of each strain obtained with formulation adjuvants. The adjuvants include, among others, a protectant (glucose) of 0.5 g, 5 ml of glycerol, 25 ml of extra virgin olive oil (dispersing agent) and 25 ml of emulsifying agent (Tween 20). Subsequently, the mixture of adjuvants and bacterial supernatant which is obtained is homogenized using a vortex for 20 minutes then placed in sterile jars, wrapped in aluminum foil and stored at 4°C for the determination of stability and for the effectiveness test on fungal strains of spoilage of the *Amélie* variety mango.

2.6. Effectiveness of formulations on mango spoilage fungal strains

The fungicidal activity of the bacterial formulations was evaluated *in vitro*, against mango spoilage fungal strains according to the modified method described by [19]. Thus, the YPGA medium was prepared and a volume of 59.4 ml of this medium was mixed with each of the bacterial formulations in sterile jars and in aseptic conditions. The bacterial formulations were mixed with YPGA medium at a concentration of 1% and then the mixture was homogenized. In addition, a consortium of biocontrol agent formulations was made by mixing 30 ml of the *Bacillus subtilis* formulation then 30 ml of the *Pseudomonas fluorescens* formulation in a sterile jar. The mixture was homogenized. Subsequently, a concentration of 1% of this consortium was mixed with a volume of 59.4 ml of YPGA medium and the mixture was also homogenized. Each mixture obtained was poured into three Petri dishes at a rate of 20 ml per dish. After the mixture solidified, a fragment of the mycelium of the same fungal was placed in the center of the box. This operation was carried out with all fungal isolates in triplicate. The control dishes were inoculated in the center of the Petri dish only with the fungi on YPGA medium without addition of the formulation or the consortium. Incubation was carried out at 30 °C for 7 days. The evolution of the confrontation of the fungal colonies and the formulation of the biocontrol agents was observed every day until the seventh day. After 7 days, the growth rate of the fungi in the plates was determined using the method of [17], then the inhibition rate was deduced according to the formula mentioned in 2.4.

3. Results

3.1. Spoilage fungal isolated from *Amélie* variety mango

Mango samples are contaminated with molds exhibiting colonies of various appearances, textures and colors. Thus, two hundred and sixteen (216) fungal isolates were isolated taking into account the resemblance of thalli and spores. Identification according to the macroscopic characteristics of the colonies (appearance, color, shape, outline, etc.) and on the basis of the microscopic characteristics of the mycelium and conidia or spores (compartmentalization of the mycelium, shape of the spores, shape of the reproductive organs, etc.) made it possible to highlight molds belonging to two (02) genera, namely *Colletotrichum* and *Lasiodiplodia*. (Table 1).

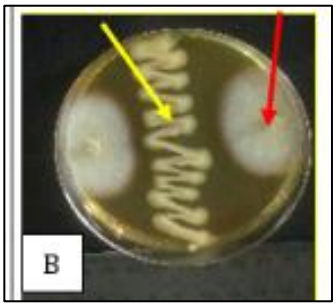
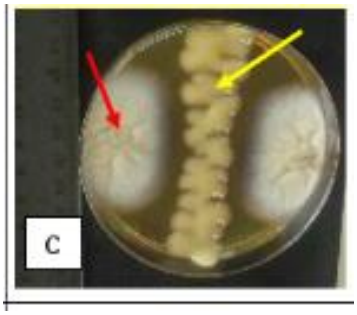
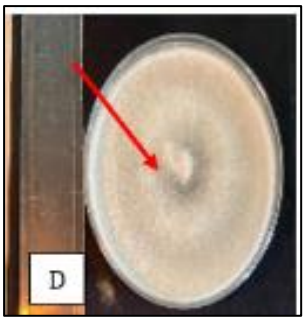
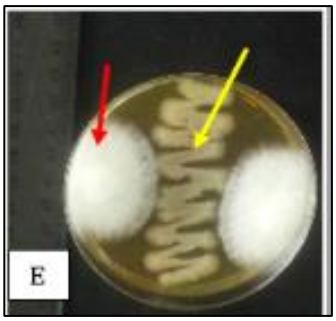
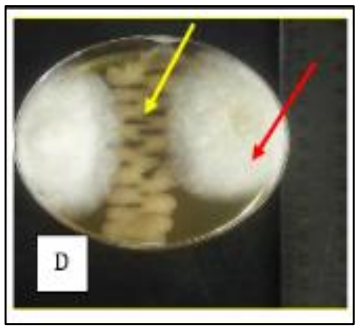
Table 1 Characteristics of isolated fungal genera

Genera	Characteristics
Colletotrichum	The colonies are fast growing and invasive with a flaky appearance. The mycelium is white in color with the presence of yellowish spores. During growth, the yellowish color diffuses all over the back of the box. Under the microscope, the hyphae are septate and branched with the presence of ellipsoidal hyaline conidia.
Lasiodiplodia	The colonies are fast growing and invasive with a cottony appearance. The mycelium, white at the start, turns black at the end. During growth, the blackish color diffuses over the entire back of the box. Under the microscope, the hyphae are septate with the presence of cylindrical hyaline conidia..

3.2. Antagonistic activities of strains of *Bacillus subtilis* and *Pseudomonas fluorescens*

The two bacterial strains biocontrol agents *Bacillus subtilis* and *Pseudomonas fluorescens* showed their inhibition capacity on the isolated strains of the two fungal genera. Table 2 highlights the inhibition rates of *Colletotrichum* and *Lasiodiplodia* affecting the *Amélie* variety mango, by strains of *Bacillus subtilis* and *Pseudomonas fluorescens*. Both strains inhibited the growth of both fungal isolates. The highest inhibition rate observed was with the *Bacillus subtilis* strain which showed a reduction of 73.54 ± 9.76 for *Colletotrichum* and a reduction of 66.82 ± 0.27 for *Lasiodiplodia*. The inhibition rates of the *Pseudomonas fluorescens* strain were estimated at 65.62 ± 1.08 for *Colletotrichum* and 64.58 ± 2.11 for *Lasiodiplodia*. The *Bacillus subtilis* strain was found to be more effective than the *Pseudomonas fluorescens* strain.

Table 2 Inhibition of fungal isolates by *Bacillus subtilis* and *Pseudomonas fluorescens*

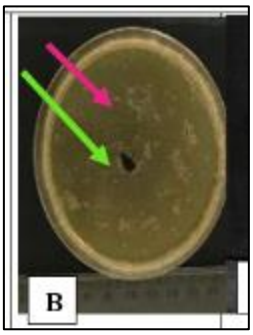
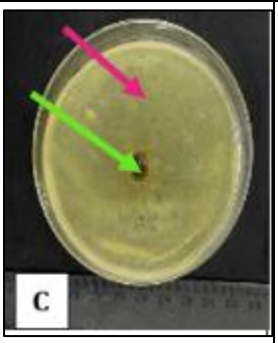
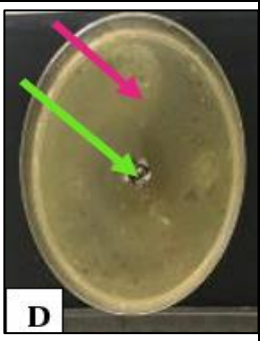
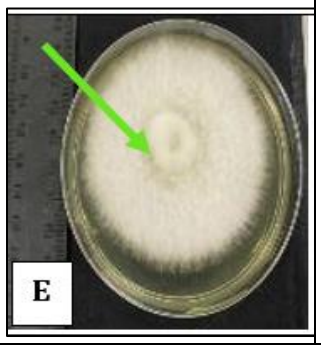
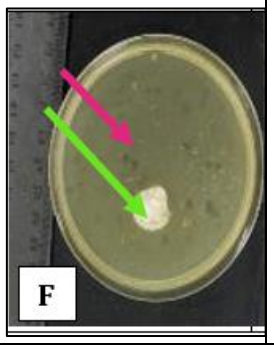
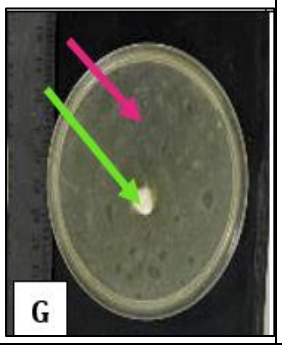
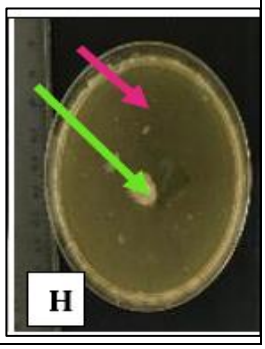
Fungal isolates	**Control**	***Bacillus subtilis***	***Pseudomonas fluorescens***
Lasiodiplodia			
Colletotrichum			

(A): control surface *Lasiodiplodia*; **(B)** and **(C):** surface of confrontation tests with *Bacillus subtilis* and *Pseudomonas fluorescens* for *Lasiodiplodia*; **(D)**: control surface *Colletotrichum*; **(E)** and (**D)**: Surface area of inhibition tests with *Bacillus subtilis* and *Pseudomonas fluorescens* for *Colletotrichum*. → : arrow indicating the biocontrol agent; → : arrow indicating the fungal

3.3. Efficacy of formulations on mango spoilage fungal strains

Liquid formulations of biological control agents based on *Bacillus subtilis* and *Pseudomonas fluorescens* as well as their consortium have shown their inhibition capacity on strains of the two isolated fungal genera. Table 3 shows the inhibition rates of *Colletotrichum* and *Lasiodiplodia* affecting the *Amélie* variety mango, by the action of bacterial formulations. The liquid formulations and their consortium inhibited the growth of both fungal isolates. The highest rate of inhibition observed was with the *Bacillus subtilis* formulation which showed a reduction of 89.06 ± 1.00 for *Lasiodiplodia*. The consortium reduction rates of bacterial formulations were increased to 88.97 ± 3.10 for *Lasiodiplodia* and 87.73 ± 3.81 for *Colletotrichum*. *Bacillus subtilis* formulation was found to be more effective on *Lasiodiplodia* and the consortium of both formulations proved to be more effective on *Colletotrichum***.**

Table 3 Inhibition of fungal isolates by formulations of *Bacillus subtilis*, *Pseudomonas fluorescens* and their consortium

Fungal isolates	**Control**	**Formulation of *Bacillus subtilis***	**Formulation of *Pseudomonas fluorescens***	**Consortium (*Bacillus subtilis+ Pseudomonas fluorescens)***
Lasiodiplodia				
Colletotrichum				

(A): control surface Lasiodiplodia sp. ; **(B), (C)** and **(D):** surface of the respective comparison tests with the formulation of *Bacillus subtilis*, *Pseudomonas fluorescens* and their consortium for *Lasiodiplodia*; **(E)**: control surface *Colletotrichum*; **(F), (G)** and **(H):** Surface area of the respective inhibition tests with the formulation of *Bacillus subtilis*, *Pseudomonas fluorescens* and their consortium for *Colletotrichum* sp.

: arrow indicating bacterial formulation; : arrow indicating fungal

4. Discussion

The general objective of this study was to reduce post-harvest losses of the *Amélie* variety mango using the formulation of two biocontrol agents (*Pseudomonas fluorescens* and *Bacillus subtilis*) against spoilage fungal germs. The results obtained following the phenotypic identification of fungal strains isolated from mangoes showed that a diversity of molds are at the origin of the post-harvest deterioration of mangoes, of the *Amélie* variety. Several presumptive genera of fungal have been identified and the most frequently isolated are *Colletotrichum* and *Lasiodiplodia*. These two (02) genera were found in all the mango samples from the orchids. These two fungal genera, mainly isolated with frequencies of 37.03% and 34.72% respectively for *Colletotrichum* and *Lasiodiplodia* would be the main agents of spoilage of mangoes. According to [20], the identification of germs responsible for post-harvest spoilage of fresh fruits in Côte d'Ivoire showed a diversity of pathogenic fungi, the main genera of which are: *Colletotrichum*, *Fusarium*, *Aspergillus*, *Phoma*, *Penicillium*, *Curvularia*, *Botryodiplodia* and *Rhizoctonia*. These fungi are known to be the cause of the spoilage of avocados, mangoes and bananas from the different production regions of Côte d'Ivoire. [21] similarly highlighted a diversity of fungi, including *Colletotrichum gloeosporioïdes*, *Botryodiplodia theobromae* and *Aspergillus niger* associated with rot of ripe mango fruits. Also, according to [22] mango in Côte d'Ivoire faces a major challenge in its conservation and export due to anthracnose, the main post-harvest disease. Identification of the causative agent of anthracnose in mango crops reveals that only the species *Colletotrichum gloeosporioïdes* is associated with this disease. *In vitro* confrontation of *Bacillus subtilis* and *Pseudomonas fluorescens* with the two fungal genera *Colletotrichum* and *Lasiodiplodia* demonstrated the inhibition capacity of these biocontrol agents against the fungal germs of mango spoilage. Their effectiveness could be explained by the ability of these biocontrol agents to produce antifungal compounds hindering mold growth after harvest. According to the work carried out by [23] cited by [19], *Pseudomonas fluorescens* produces in the stationary phase of growth, various metabolites with strong antifungal properties, including phenazines, pyrrolnitrin, pyoluteorin and 2,4-diacetylphloroglucinol (2,4-DAPG) capable of prevent the growth of pathogens. These metabolites act by altering the germination, growth and/or sporulation of the pathogen, modifying

the appearance of colonies and producing specific forms such as pseudo parenchyma. *Bacillus subtilis* can have an inhibitory effect on pathogens through the production of lipopeptides such as surfactins, iturins, fengycins, bacillomycins and subtilins. These compounds would have an effect on the walls and on the mycelial growth of pathogens [24]. These results are consistent with those of [25]who combated fungal contaminants in pineapple using *Bacillus subtilis* GA1, *Pseudomonas fluorescens* CI and *Pseudomonas fluorescens* F19. This made it possible to preserve the pineapple fruits for a period of more than 14 days. This observation is further corroborated by [10] which inhibited zucchini spoilage germs such as *Candida* sp., *Aspergillus* sp. and *Phytophthora* sp. by *Pseudomonas fluorescens* F19. These antifungal properties of *Pseudomonas fluorescens* F19 made it possible to extend the shelf life of Coucourzelle variety zucchini over a period of 10 days. The inhibition rates of biocontrol agents against the main spoilage molds of mangoes were greater than 60%. *Bacillus subtilis* therefore gave inhibition rates of between 66.82% and 73.54%. As for *Pseudomonas fluorescens*, the inhibition rate increased from 64.58% to 65.62%. These results are consistent with those obtained by [26], during their work on the conservation of pineapple. They noticed that *Bacillus subtilis* GA1 inhibited *Geotrichum candidum* with an inhibition rate of 66.67% and *Pseudomonas fluorescens* CI prevented the growth of *Aspergillus aculeatus* at a rate of 63.17%. Similarly, [16], during their work on mango conservation observed that *Bacillus subtilis* GA1 inhibited the growth of *Colletotrichum* sp. at an inhibition rate of 60.5%. The results of *in vitro* antagonist tests carried out with the two formulated products as well as their mixture demonstrated positive effects in reducing the growth of *Colletotrichum* and *Lasiodiplodia.*These results indicate that the metabolites present in these formulations could offer a good capacity to protect fruits against the germs responsible for the spoilage of mangoes. The inhibition rates of the *Bacillus subtilis* formulation were between 76.63% and 89.06% while those of *Pseudomonas fluorescens* ranged from 78.21% to 83.57%. Regarding the inhibition rates of the consortium formulation, they were between 87.73% and 88.97%. This work is consistent with that of [19] who produced two liquid formulations from *Pseudomonas fluorescens* and *Bacillus subtilis* to fight against bacteriosis (*Xanthomonas citri pv. Mangifereaindicae*) of the cashew tree in the greenhouse. The capacity of the two formulated products to protect cashew plants against bacteriosis was 80.95% and 73.80% respectively for the formulations of *Pseudomonas fluorescens* and *Bacillus subtilis*. In the same vein, the work of [27] on the antibacterial activity of the formulation based on *Pseudomonas fluorescens* strain SP007S against *Pectobacterium carotovorum*, the bacteria responsible for soft rot of kale, reduced kale disease by 65.02%.

5. Conclusion

This present study made it possible to contribute to the fight against spoilage fungi of the *Amélie* variety mango with a view to reducing post-harvest losses. Thus, two (02) genera of fungi, namely *Colletotrichum* and *Lasiodiplodia*, were identified as the main germs of mango spoilage and those most incriminated in spoilage. Liquid formulations made from *Bacillus subtilis* and *Pseudomonas fluorescens* made it possible to inhibit the isolated fungal strains responsible for damage to the Amélie mango. Therefore, *Bacillus subtilis* and *Pseudomons fluorescens* could be used as biopesticides in the fight against mango pathogens.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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