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New Bacterial Agents to Limit *Colletotrichum gloeosporioides* Development on Mango

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Abstract

Mango anthracnose disease forms typical irregular-shaped black necrotic spots on the fruit peel of mature fruit and is caused by *Colletotrichum gloeosporioides*. In order to improve the disease control with a limited use of fungicides, new microbial agents able to limit the growth of the pathogen were searched in the indigenous natural flora of mango surface. In order to find a suitable biocontrol agent, a screening was applied to 305 epiphytic bacteria isolated from the carposphere of 17 mango cultivars sampled from eight locations on Reunion Island. The screening approach involved a first step based on the ability of the isolates to form a biofilm, to grow under fruit storage conditions, and to interfere with the development of *C. gloeosporioides*. In a second step, the capability of selected isolates to limit *C. gloeosporioides in vitro* mycelial growth and conidia germination was assessed and species identified. The most effective bacteria belonged to the *Enterobacter*, *Pantoea*, *Kosakonia* and *Leuconostoc* genera, but for some of them, their safe use has to be demonstrated. Efficacy *in vivo*, performed on wounded mature mango fruit, was limited, probably because of the wounding inoculation strategy favoring the pathogen. Future biocontrol treatments should focus on preharvest applications to enhance the protective benefit.

Keywords

Anthracnose, Epiphytic Bacteria, Antagonism

1. Introduction

Mango occupies a very important place in culinary traditions and is of economic

importance in some tropical and subtropical countries. Mango production currently ranks seventh in global fruit production with 50.65 million tons in 2017 [1]. For commercial purpose, the high-end fruit should be free of external damage, decay, bruises, and latex or sap injury.

Postharvest diseases such as anthracnose reduce fruit commercial quality and lead to significant losses. Black spots mostly develop during post-harvest stages and lead to fruit decay. Mango anthracnose is caused by *Colletotrichum gloeosporioides* fungal species and remains the major mango postharvest disease in Reunion Island [2]. Mature fruit affected by anthracnose develop sunken, prominent, dark brown to black decay spots before or after picking. The development of fungal mycelia under the mango skin leads to the necrosis of the epidermal cells, which makes the fruit unmarketable. The disease symptoms only appear after fruit ripening, although mango contamination occurs by splashing conidia during the rainy period and is followed by an appressorial (quiescent form) phase which lasts until favourable germination conditions [3].

C. gloeosporioides sensu lato is responsible for fruit diseases, referred to as "anthracnose", on many other tropical fruits including banana (*Musa* spp.) [4] [5] [6], avocado (*Persea americana*) [7] [8], papaya (*Carica papaya*) [9] [10] guava (*Psidium guajava*) [11] [12], passion fruit (*Passiflora* spp.) [13] [14], dragon fruit (*Hylocereus undatus*) [15] and others [5] [6] [16] [17].

Despite the high efficacy of commercial fungicides against *C. gloeosporioides*, the increasing emergence of fungicide-resistant isolates has been reported [18] [19] [20] [21]. Therefore, several fungicides have been withdrawn from the market due to pathogen resistance. Moreover, fungicides are responsible for environmental and public health hazards [22]. On the other hand, there is a growing public demand for organically produced crops and recent European regulations impose the decrease the Maximal Residue Levels (MRL) [23]. Therefore, there is a need for the development of new technologies and methods that are alternative to synthetic fungicides for better fruit postharvest disease control.

Among alternative approaches to fungicides, biological control of postharvest diseases of mango by microbial antagonists has been under investigation for decades. Both bacteria and fungi have been isolated and characterized as potential biocontrol agents for fruit post-harvest diseases. They can inhibit the proliferation of pathogens both *in vitro* and *in vivo*. Efficiency and mode of action of these antagonists against *Colletotrichum* species complex have been particularly investigated [22] [23].

Despite extensive research on anthracnose biocontrol, only few commercial products are available and used. This is due to a lack of efficacy of biocontrol agents when it comes to large scale *in vivo* trials [22] [23] [24]. The inability of biocontrol agents to colonize fruit surface has been hypothesized. In most cases, postharvest disease biocontrol agents are applied after harvest. They have then to compete with the natural epiphytic microflora to colonize fruit surface. One solution to limit this effect, beside adapting the biocontrol agent to fruit surface

conditions, would be to select microflora from the same environment, thereby limiting adaptation requirement to a different niche. Efficient biocontrol agents have to persist in a wide range of environmental conditions [25] [26] [27].

Most fungal biocontrol agents screened against mango anthracnose were isolated from mango environment, either orchard soil [28] [29] [30] or fruit. The yeasts *Debaryomyces nepalensis* [30] and *Metschnikowia pulcherrima* [31] [32] reduced the severity of mango anthracnose symptoms. The yeast *Cryptococcus laurentii* was isolated from mango surface and its antagonistic activity relies on several mechanisms such as competition for nutrients and space [33]. *Meyerozyma caribbica* also isolated from mango fruit showed competition for space, competition for nutrients, parasitism and lytic enzyme production [33]. The mechanism of action of *Trichoderma asperellum* against the phytopathogen was parasitism, through the production of glucanase, cellulase and chitinase [34].

Contrarily to fungal biocontrol agents, bacteria selected to biocontrol mango anthracnose come from a wide range of environments: plant isolate collections [35], soil [36], green banana surface [37] or mango leaf [38] [39]. The spore-forming bacteria *Bacillus thuringiensis*, *Bacillus pumilus* [36], *Bacillus licheniformis* [40] [41] and *Bacillus amyloliquefaciens* [37] produce antifungal metabolites which mediate a direct *in vitro* inhibition of phytopathogen growth, whereas *Pseudomonas fluorescens* induces plant defences, especially by production of fungal cell wall lytic enzymes [35].

Bacterial biocontrol agents present several advantages comparatively to fungi [42]. They are easier to grow and many of them, especially lactic acid bacteria (LAB), cope with food safety regulation criteria [43]. In addition, LAB do not form spores and can easily be inactivated during fruit processing. Therefore, the first objective of this study was to isolate potential biocontrol agents, particularly targeting LAB, from the surface of mature mango locally harvested, to investigate the *in vitro* interactions of mango epiphytic bacteria with *C. gloeosporioides*, and to assay the *in vivo* activity in order to evaluate the impact on disease development on fruit.

2. Materials and Methods

2.1. Isolation of Bacteria and Culture Conditions

Mangoes were harvested from eight locations of Reunion Island, over a period of 10 weeks (**Table 1**). Reunion Island is characterized by a tropical climate, with average annual temperature of 25˚C and ferrallitic soils [44]. Annual rainfall largely varies depending on the location: from 500 - 1000 mm for West locations like Saint Paul, Grand Fond and Savana, to 1250 - 2000 mm for Bassin Plat and 2000 - 3000 mm for East locations like Cambuston, Quartier Français and Sainte Suzanne [45]. Mature mangoes, from 17 cultivars, were selected and manipulated without direct hand contact. Bacteria were collected from the fruit surface with a sterile cotton swab soaked in 10 mL of buffered peptone water [46]. By using a sterile pipette, the peptone water was collected and poured into a sterile

Table 1. Mango sampling and bacteria isolation. Both number and name of isolates are indicated depending on the medium of isolation, either MRS at 30˚C or NM at 12˚C.

Location	Cultivar	Sampling date	Number of MRS isolates	MRS isolate names	Number of NM isolates	NM isolate names
Saint Paul - organic	Cogshall	12/11/15	1	H001	5	H002 to H006
Saint Paul - organic	Cogshall	02/12/15	7	H008, H009, H014 to H018	5	H020 to H024
Saint Paul - organic	Cogshall derived	02/12/15	1	H010	0	
Saint Paul - organic	David Haden	02/12/15	2	H012, H013	1	H025
Saint Paul - organic	José derived	02/12/15	2	H011, H019	1	H026
Bassin Plat	Cogshall	11/01/16	0		2	H027, H028
Bassin Plat	Irwin	11/01/16	4	H306, H309 to H311	2	H029, H296
Bassin Plat	Kensington Pride	11/01/16	0		3	H030, H031, H032
Bassin Plat	Tommy Atkins	11/01/16	1	H037	4	H033 to H036
Grand Fond	Kent	15/01/16	2	H050, H302	14	H086 to H098, H292, H293
Grand Fond	Nam DokMaï	15/01/16	8	H053 to H056, H059 to H062	9	H109 to H117
Grand Fond	Tommy Atkins	15/01/16	4	H057, H303 to H305	12	H099 to H108, H294, H295
Bassin Plat	Cogshall	18/01/16	5	H080 to H084	8	H174 to H181
Bassin Plat	Irwin	18/01/16	4	H067 to H070	12	H132 to H143
Bassin Plat	Kensington Pride	18/01/16	8	H058, H063 to H066, H071, H307, H308	17	H118 to H131, H297 to H299
Bassin Plat	Nam DokMaï	18/01/16	10	H051, H052, H072 to H079	17	H144 to H160
Bassin Plat	Tommy Atkins	18/01/16	12	H040 to H049, H038, H039	13	H161 to H173
Grand Fond	Heidi	23/01/16	0		23	H182 to H204
Saint Paul	Auguste	29/01/16	0		7	H205 to H211
Saint Paul	Heidi	29/01/16	0		3	H212 to H214
Saint Paul	José	29/01/16	1	H085	13	H219 to H231
Cambuston	José	08/02/16	7	H247 to H253	11	H271 to H281
Quartier Français	José	08/02/16	11	H236 to H246	7	H264 to H270
Sainte Suzanne	José	08/02/16	3	H254 to H256	2	H282, H283
Savana	José	08/02/16	4	H232 to H235	7	H257 to H263
Saint Paul - organic	Caro	17/02/16	1	H300	3	H284 to H286

Continued

Saint Paul - organic	Cécile	17/02/16	0		1	H287
Saint Paul - organic	Pierrefontaine	17/02/16	0		2	H288, H289
Saint Paul - organic	Ticroix	17/02/16	0		1	H290
Saint Paul - organic	Valencia	17/02/16	1	H301	1	H291

tube. The microbial solution obtained was streaked over MRS (de Man, Rogosa and Sharpe) agar and Nutritive Medium (NM) plates and placed at 30˚C for 72 h and at 12˚C for 10 days, respectively. Colonies with different aspects were isolated on the same growth medium after microscopy examination. Isolated strains were stored at −80˚C in 20% glycerol.

Bacillus subtilis AvoGreen was used as a reference biocontrol strain [38].

Before use, bacteria were cultivated in broth, either MRS or NM depending on the isolation medium, at 25˚C during 72 h in an incubator with agitation at 100 rpm.

2.2. *Colletotrichum* spp. Cultivation

The MUCL 43868 strain of *C. gloeosporioides* (Penzig) from the pathogen collection of the Catholic University of Leuven (Leuven, Belgium) was used and cultivated on potato dextrose agar (PDA) medium at 27.5˚C. This strain was isolated from Mexican mangoes by GL Hennebert [47]. Strains CG Aust Mango 3-3 and CG Avocado 23-703 were obtained from the collection of Pr. Korsten.

An inoculum of *C. gloeosporioides* was grown on PDA plates over 10 days at 27˚C. Conidia were collected by pouring 10 mL of sterilized buffered peptone water on the plates and recovered by pipetting and adjusted to a final concentration of 10^5 conidia per mL using a Malassez cell.

2.3. Growth at Different Temperatures and Biofilm Formation

In a 96-well microplate, 180 µL of isolation medium broth and 20 µL of a 72 h bacterial culture were deposited. Each bacterial culture was loaded into 3 wells. Controls corresponded to 200 µL of isolation medium broth, distributed in 3 wells. Six batches of microplates were prepared and placed in incubator, with a lid on, at 12˚C, 25˚C, 30˚C, 37˚C and 42˚C, over 72 h without agitation. The absorbance was read for each microplate three times a day until 72 h of incubation with the microplate reader. For each time point, the plate was shaken for 15s, then the reading was performed at a wavelength of 600 nm and the collected OD was the mean of 10 reads of the same well. For each well, the growth curve was plotted over time and maximal growth rate (μ_{max}) was determined from OD slope over the growth phase. The optimal growth temperature was defined as the

temperature leading to the highest μ_{max}.

After 72 h, each microplate incubated at 25˚C was emptied and rinsed in clear water, then 125 µL of a 0.1% of crystal violet solution was added in each well. After 10 min, the content of the wells was dropped off, rinsed again with clear water and then dried at open air. A volume of 200 µL of 30% acetic acid solution was added and 125 µL were taken off and poured in a new microplate. The microplate was read on a 550 - 680 nm wavelength range on the microplate reader and the wavelength rendering the maximal absorbance (590 nm) was chosen for the exploitation of the results. Biofilm strength is given by the ratio of absorbance between the bacteria and the control wells.

2.4. Identification of Isolates

DNA extraction from bacterial strains was performed using the InstaGene Matrix commercial kit (Bio-Rad Laboratories, Hercules, CA, USA) [48]. For the amplification of the 16S rDNA region, the DNA primer pair was used: FD1-mod 5'-3': AGAGTTTGATCHTGGCTCAG and RD1-mod 5'-3': GGMTACCTTGTTACGAYTTC [49]. The reaction volume was composed of 5 µl of purified DNA and 45 µl of a mixed solution composed of: 10 µl 5 × PCR buffer (Green Go Taq), 1 µl dNTP mixture (10 mM), 4 µl $MgCl_2$ (25 mM), 1.0 µl of each primers (FD1-mod and RD1-mod at 1 µM), 0.25 µl enzyme Go Taq DNA polymerase (5 U/µL) and qs of water. PCR amplification was carried out using Applied BiosystemsVeriti ™ Thermal Cycler. The thermal cycling program was: 3 min at 94˚C, followed by 35 cycles of [40 sec at 94˚C, 40 sec at 55˚C, 60 sec at 72˚C], and further for a time extension of 10 min at 72˚C. The quality of the amplification products was analysed on 2% TAE agarose gels after electrophoresis for 90 min at 110 V and staining with ethidium bromide.

2.5. *In Vitro* Colletotrichum Inhibition Assay

2.5.1. Plate Assay for Mycelial Growth Inhibition

To assay mycelial growth inhibition, *C. gloeosporioides* MUCL 43868, CG Aust Mango 3-3 and CG Avocado 23-703 strains were used. Mycelium (0.5 mm × 0.5 mm) was spotted in the middle of a plate containing PDA medium and incubated for 21 days at 30˚C. Bacterial isolates were grown on nutrient agar (NA) incubated at 30˚C for 48 h. A suspension was created by gently scraping the bacterial lawn from NA. The OD at 600 nm of the suspension was adjusted to 1 unit (per mL) as assessed with PowerWave™ microplate spectrophotometer (BioTek). A volume of 100 µL was used for inoculation.

Two mycelium pieces were deposited on opposite sides of the PDA plates. The bacterial strains were inoculated as a central streak 48 h after inoculation of the fungus. Plates were incubated for 10 - 15 days at 30˚C, until the complete colonization of the plate surface by the fungus in the control plate (without bacteria). Thereafter, diameters of mycelium were measured to calculate a percentage of radial inhibition. Percentage of inhibition was calculated from the diameter of

Colletotrichum colony in the presence of an isolate compared to the diameter without any inhibitor (0% of inhibition).

2.5.2. Inhibition of the Germination of Conidia

A spore suspension of MUCL 43868 strain of *C. gloeosporioides* was prepared by placing 10 mL of peptone water (or sterile distilled water) in the Petri dish containing a 15-day fungus culture. After filtration, the concentration of spores was adjusted to 5 log spores/mL using a Malassez cell.

Conidia germination inhibitory bacterial activity was evaluated by microscopy on special sterile slides. The first step was the deposit of 100 µL of melted PDA medium on the slides, followed by drying under a laminar flow hood for 1 hour. To carry out the test, 10 µL of spore suspension were deposited on the slide and 10 µL of each bacterial strain were added. The slide was then incubated at 27˚C inside a Petri dish lined with a double layer of moistened filter paper. Each treatment was repeated twice. The control corresponded to the suspension of spores in the absence of bacteria.

A conidiospore was considered to have germinated when a germinating tube of at least half the length of the conidiospore was observed under optical microscope. The qualitative data for the inhibition of germination of *C. gloeosporioides* MUCL 43868 were described as; (+): the germination of less than 25% of conidia was inhibited; (++): the germination of 25% - 50% of conidia was inhibited; (+++): the germination of 50% - 75% of conidia was inhibited; (++++): more than 75% of conidia germination was inhibited. Negative control showed no inhibition of conidia germination which corresponded to a germination rate close to 100%.

2.6. *In Vivo* Examination of the Severity of Anthracnose

The ˚C. *gloeosporioides* strain MUCL 43868 was cultivated in Petri dishes for 21 days on PDA solid medium at 27.5˚C in the dark.

Mangoes cv. José were harvested and treated on the same day. A batch of 36 mangoes cv. José collected from the same orchard and with same maturity level (yellow point) was used. Fruit with no disease symptoms were selected, and the surface to be inoculated was washed with 70% ethanol and air-dried at room temperature. Subsequently, fruit inoculation for curative treatment was performed according to [50] by uniformly wounding (a cross: 2 mm deep and 10 mm wide) a relatively flat area in the middle of the fruit with a sterilized cork-borer and inoculating it with 20 µL of a spore suspension of *C. gloeosporioides* (10^5 spores/mL). To ensure that anthracnose development was due to MUCL 43868, the peels from non-inoculated and inoculated fruits were placed on PDA amended with chloramphenicol and left for 8 days at 25˚C. The identification of the re-isolated fungi was based on morphological criteria. After fungal inoculation, fruits were maintained at room temperature for 24 h (21˚C, 85% relative humidity).

Bacteria *Enterobacter* sp. H222 and *Leuconostoc mesenteroides* H255 were

separately grown in nutrient broth for 3 days at 27.5˚C in a nutrient broth. Two suspensions corresponding to OD at 600 nm of 0.5 and 1.0 were prepared for each isolate.

From all 36 mangoes inoculated with *C. gloeosporioides*, six fruits were submerged in distilled water, to be used as non-treated positive controls. Three treatments were used to assess the impact of bacteria on anthracnose development. The thirty remaining mangoes were separated into three batches of 10 fruits corresponding to [H222], [H255], and [H222 + H255] treatments. Pre-inoculated mangoes were immersed in each bacterial suspension for two minutes. Fruit were stored at 20˚C with high relative humidity. After 10 days of storage, black spots on the surface of mangoes were measured using a digital caliper.

The experiment was repeated twice with independent batches of mango.

2.7. Statistical Analysis

XLSTATsoftware (Addinsoft, Paris, France) was used for statistical analysis. K-means clustering and hierarchical clustering analysis (HCA) were performed with maximal growth rate, optimal growth temperature, ability to form biofilm and inhibition of mycelial growth as variables and isolates as observations. Variables were centred and normalized, and Euclidian distances were used. For k-means clustering, trace (W), *i.e.* pooled SSCP matrix, was used as classification criterion. For HCA, Ward aggregation method was used. Dendrogram was built through the Ward's minimal distance algorithm. To compare inhibition of fungal growth, ANOVA was used with the REGWQ test and the bilateral Dunnet's test was used for *in vivo* assay.

3. Results and Discussion

3.1. Isolation and Phenotypic Characterization of Bacteria

Bacteria were collected from 17 mango cultivars grown in eight locations on Reunion Island in order to cover the widest diversity of epiphytic mango bacteria available locally.

As mangoes are generally stored under refrigerated conditions, isolation of bacteria was performed from NM incubated at 12˚C to select psychrotrophic bacteria. Colonies of LAB were also recovered as these bacteria have a long history of use in food and some of them are already used to preserve foods. From mango surface, 305 bacteria were isolated: 99 were isolated from MRS, and 206 from NM (Table 1).

The most appropriate candidates for biocontrol of mango anthracnose were then selected without prejudice of the bacterial species and through a funnel-shaped approach.

The maximal growth rate, the optimal temperature for growth, the ability to form biofilms and the level of inhibition of *C. gloeosporioides* MUCL 43868 mycelial growth were determined for the 305 isolates. Two classification methods,

k-means and HCA, were applied, resulting in similar results. Isolates were clustered into six classes, leading to an inter-classes' variance of 79% of the total variance (Table 2 and Figure 1). This high inter-classes' variance value implies that classes were clearly defined and well differentiated.

The most homogeneous class (class 6), *i.e.* with the lowest intra-class variance, gathered 24 isolates, characterized by low optimal growth temperature and low inhibition level. Class 5 included the highest number of isolates (133) and was also characterized by a low inhibition level of mycelial growth. Class 4, which gathered 18 isolates, harboured the same characteristics, but with the highest ability to form biofilm. Classes 1, 2 and 3 were the most interesting regarding the inhibition of mycelial growth, with an inhibition level of the gravity centre of 18.3%, 25.4% and 17.0% respectively. Those three classes differed by the number of isolates, class 1 being the largest. Class 1 was characterized by the highest μmax and optimal growth temperature. Class 2 was characterized by the highest mycelial growth inhibition level, an intermediate optimal growth temperature of 28.0˚C and the lowest ability to form biofilm. Class 3 gathered psychrophilic bacteria, with the highest ability to form biofilms. Among the three interesting classes, inhibition level was the lowest in class 3.

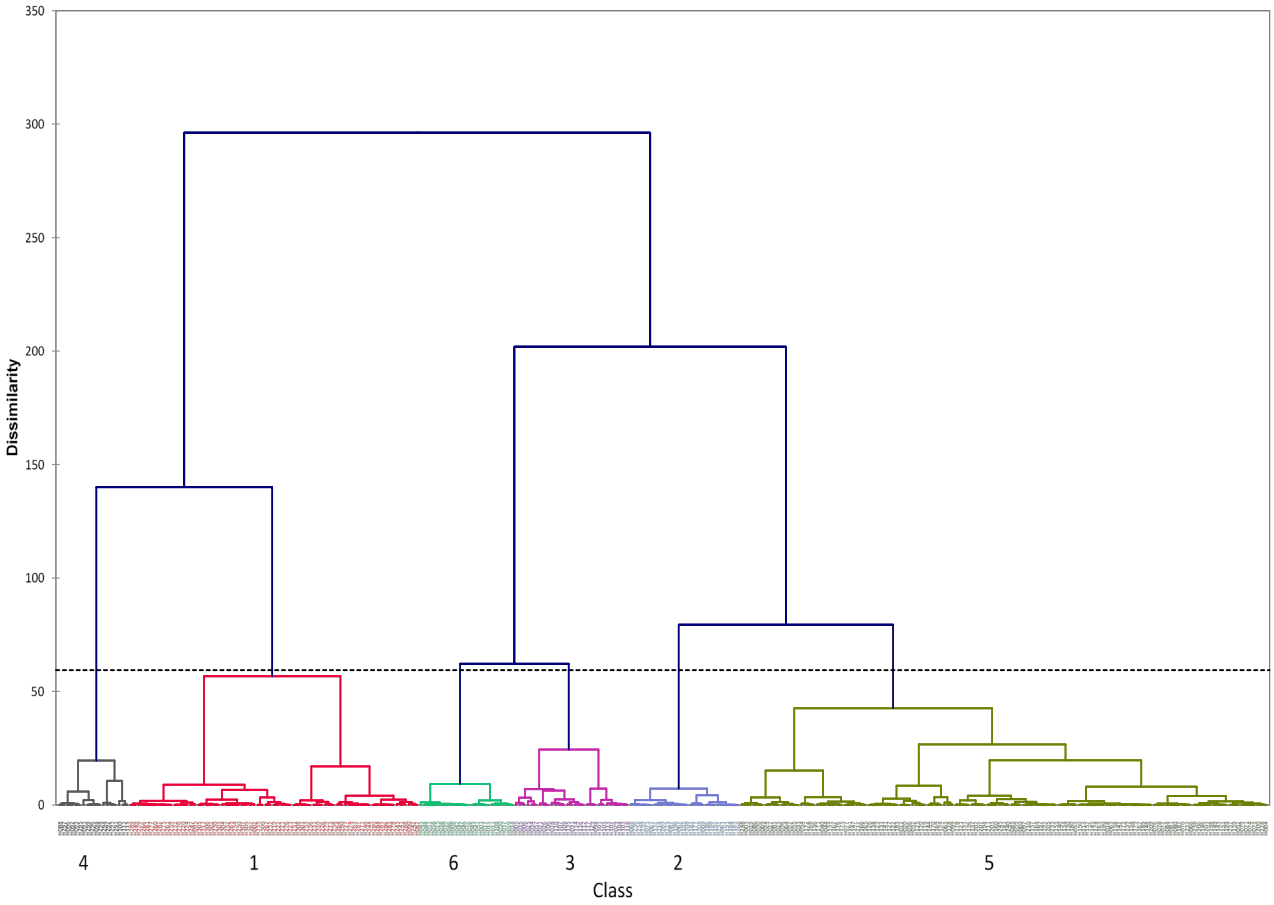

Figure 1. Dendrogram of 305 isolates classified into six classes by hierarchical clustering analysis (HCA) based on the variables "maximal growth rate", "optimal growth temperature", "ability to form biofilm" and "inhibition of mycelial growth".

Table 2. Characteristics of classes of isolates, according to maximal growth rate (μmax), optimal growth temperature (˚C), biofilm strength and inhibition activity (%) against *Colletotrichum gloeosporioides* MUCL 43868 hyphal growth.

Class	1	2	3	4	5	6
Number of isolates	73	28	29	18	133	24
Intra-class variance	14.1	19.7	15.5	28.2	23.9	0.7
Mean distance to gravity centre	3.6	3.7	3.1	4.8	4.1	0.7
Gravity centre μmax (h-1)	0.36	0.10	0.22	0.31	0.13	0.10
Gravity centre optimal growth temperature (˚C)	34.3	28.0	12.0	35.4	30.1	12.0
Gravity centre biofilm strength	2.3	1.8	9.1	13.7	3.1	2.2
Gravity centre inhibition level (%)	18.3	25.4	17.0	11.5	12.1	11.6

3.2. Selection and Identification of Inhibitors of *Colletotrichum* Development

From the three classes showing the highest inhibition ability, 26 isolates were selected and identified by sequencing of the chromosomal region encoding 16S rRNA (Table 3). From the first class, 11 isolates were selected, representing 15% of the class size, whereas 12 isolates (43%) were selected from class 2 and 3 (10%) from class 3. No isolates from class 4 were selected in spite of the highest ability to form biofilm which is considered as an advantage to help the antagonist to colonize fruit surface [26], because of lower ability to inhibit the fungal pathogen.

Among those, 19 isolates were identified as Enterobacterales: eight belonged to the genus *Enterobacter* and seven were identified as *Kosakonia cowanii*. The other genera were *Pantoea* and *Serratia*. Besides, one isolate was identified as a *Microbacteriaceae*, *Curtobacterium luteum*, and two *Staphylococcus* species were represented. More interestingly because of their safety for food use, three isolates were identified as *L. mesenteroides* and one as *Gluconobacter* sp.

Most of the species hereby identified were previously described as part of plant microbiomes. *Enterobacter*, *Pantoea* and *Curtobacterium* were identified from salad leaves [51]. *Enterobacter*, *Pantoea*, *Leuconostoc* and *Curtobacterium* were identified on tomato fruit or leaves surface [52] [53] [54]. The same genera, plus *Staphylococcus* and *Gluconobacter* were identified on mango tree leaves [55]. *Gluconobacter* was identified on grape surface, but *Sphingomonas* was the most abundant genus in this niche [56]. Interestingly, *Kosakonia cowanii* was here for the first time reported as isolated from carposphere. *K. cowanii* is the type species of *Kosakonia*, and was isolated from clinical and environmental samples, especially soil and trees [57].

Serratia marcescens is essentially studied for its role in nosocomial infections and for chitinase production [58] [59]. For these reasons, isolates from the present work were not further studied. Four of the *Enterobacter* isolates belong to the *Enterobacter cloacae* complex, which gathers 12 species which cannot be easily differentiated from 16S rRNA coding region sequence [60] [61]. Many

Table 3. Isolate identification and inhibition of *C. gloeosporioides* MUCL 43868 hyphal growth (%) and conidia germination.

Class	Isolates	Identification	Hyphal growth inhibition, %[1]		Conidia germination inhibition[2]	
3	H028	*Enterobacter* sp.	31.7	±	9.9	++++
2	H061	*Enterobacter* sp.	32.4	±	23.9	+
2	H068	*Enterobacter* sp.	27.7	±	6.1	++++
1	H221	*Enterobacter* sp.	25.9	±	4.0	+
1	H222	*Enterobacter cloacae* complex	37.4	±	7.8	++++
1	H223	*E. cloacae* complex	26.7	±	6.6	++++
2	H232	*E. cloacae* complex	23.0	±	4.8	++++
1	H267	*E. cloacae* complex	22.0	±	12.8	+
1	H219	*Kosakonia cowanii*	52.0	±	21.2	+
2	H185	*K. cowanii*	43.3	±	18.1	+
2	H182	*K. cowanii*	33.9	±	9.0	++
2	H184	*K. cowanii*	32.4	±	7.1	++
2	H192	*K. cowanii*	30.5	±	3.1	++
2	H191	*K. cowanii*	29.4	±	5.0	+++
2	H188	*K. cowanii*	27.3	±	6.3	++
2	H186	*Pantoea dispersa*	30.2	±	2.8	++
1	H272	*Pantoea* sp.	24.9	±	0.8	+
3	H129	*Serratia* sp.	24.2	±	5.1	+
2	H177	*Serratia marcescens*	22.7	±	6.9	++
3	H117	*Curtobacterium* sp.	23.3	±	11.6	+
1	H311	*Gluconobacter* sp.	20.4	±	6.1	+
1	H270	*Leuconostoc mesenteroides*	21.1	±	4.7	+
1	H255	*L. mesenteroides*	20.6	±	8.8	++++
1	H305	*L. mesenteroides*	16.8	±	6.1	+
2	H268	*Staphylococcus fleurettii*	14.5	±	14.3	+
1	H300	*Staphylococcus hominis*	27.5	±	11.5	+

[1]mean ± standard deviation; [2](+): the germination of less than 25% of conidia was inhibited; (++): the germination of 25% - 50% of conidia was inhibited; (+++): the germination of 50% - 75% of conidia was inhibited; (+++): more than 75% of conidia germination was inhibited.

isolates from this complex originate from clinical samples [61] [62] [63], and their multidrug-resistance capacity raises concerns about their pathogenicity and virulence. However, many other isolates come from plant, possibly plant pathogens and isolates from this complex might play a role in biocontrol [64]. The *Pantoea* genus is widely distributed in nature and many species are described as epiphytes, endophytes or plant pathogens [65]. Many isolates, some being from the species *Pantoea dispersa*, have been used as biocontrol agents against post-harvest rots of fruit, onion or sweet potato [66]-[71]. *Pantoea agglomerans* strain CPA-2 is an effective biocontrol agent (BCA) against the major postharv-

est pathogens present on pome and citrus fruits [72] [73] [74] [75]. *K. cowanii* was classified in this new genus in 2013 from *Enterobacter* and based on genomic polymorphism analysis [55] [76]. This species can act as a plant growth promoter, especially for sugar cane [77].

Curtobacterium was found as an endophytic bacterium of many plants including rambutan fruit [78] [79] [80]. It has been studied as a biocontrol agent for fungal brown rot of plum [27].

Among other LAB, *L. mesenteroides* is described for its antifungal activities and potential use for biocontrol [81] [82]. This bacterium is commonly found in fermented foods, especially from vegetables and fruit [83] [84].

Mycelial growth inhibition was in the range 20% - 40% for most isolates, the highest inhibition being observed for two *Enterobacter* isolates, two *Kosakonia* isolates and *P. dispersa*. Complementary to hyphal growth inhibition, isolates were tested for their ability to inhibit MUCL 43868 conidia germination (**Table 3** and **Figure 2**). The results were listed in four classes according to isolate capability to inhibit germination, from low to strong inhibition [<25%, 25% - 50%, 50% - 75% and >75% of conidia inhibited]. Contrarily, conidia germination

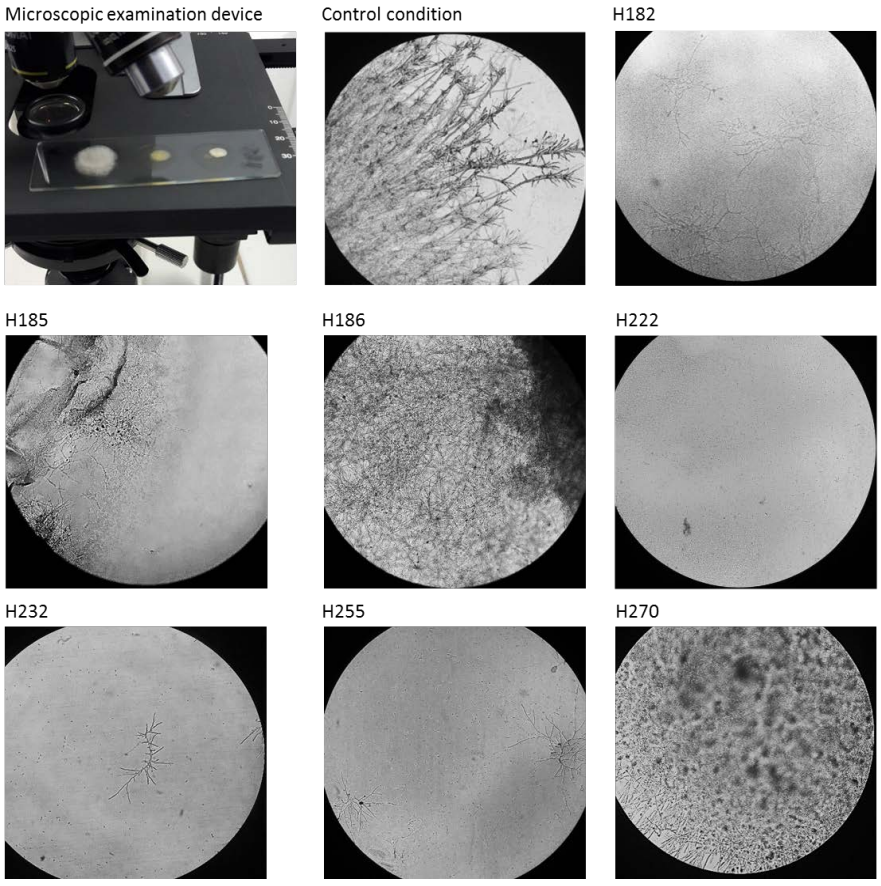

Figure 2. *In vitro* slide test of inhibition of conidial germination of *C. gloeosporioides* MUCL 43868. The device used, germination of conidia under control condition, and germination of conidia in presence of bacteria isolates H182, H185, H186, H222, H232, H255 and H270 are shown. A magnification time of 400 was used.

inhibition was clearly more marked for *Enterobacter* and *Leuconostoc* (Table 3). Six isolates inhibited the germination of more than 75% of conidia: five *Enterobacter* isolates (H028, H068, H232, H222, H223), and *L. mesenteroides* H255. The *K. cowanii* H191 isolate inhibited the germination of 50% - 75% of conidia. Six isolates, four from *K. cowanii* (H182, H184, H192, H188), *P. dispersa* H186 and *Serratia marcescens* H177 inhibited 25% - 50% of conidia. The other isolates inhibited the germination of <25% of conidia.

From these results, isolates from species exhibiting good inhibition ability were selected to further assay inhibition capacity: *Kosakonia* H182 and H191, *Pantoea* H186, *Enterobacter* H222 and H232, and *Leuconostoc* H255. *L. mesenteroides* H270 was added to this pool of isolates as it has a safe use status as a LAB. *B. subtilis* AvoGreen was used as a reference. The eight isolates were tested against the hyphal growth of two other strains of *C. gloeosporioides*, one from South Africa collected from avocado and the other one from Australian mango (Table 4). Most of the isolates showed a hyphal growth inhibition activity in the range 19.3% to 24.9%. The isolate *Kosakonia* H182 exhibited a lower inhibition activity (10.0%) on the strain CG Avocado 23-703. AvoGreen and *Enterobacter* H222 showed inhibition activities above 30.4% against the two fungal strains, and *Kosakonia* H191 a 34.9% inhibition of hyphal growth of strain CG Aust Mango 3-3.

Whatever the fungal strain assayed, *Enterobacter* H222 was clearly the most efficient.

3.3. *In Vivo* Activity

An *in vivo* assay was performed on a local variety of mango (cv. José), purposely injured and inoculated with fugal conidia. Selected bacteria, *Enterobacter* H222 and *Leuconostoc* H255, were added 24 h later and mangoes were stored at 20˚C.

Table 4. Inhibition activity (%) of *C. gloeosporioides* CG Aust Mango 3-3 and CG Avocado 23-703 hyphal growth. Different letter in a column indicates a significant difference (p-value < 0.001).

Isolate	CG Aust Mango 3-3			CG Avocado 23-703		
	Hyphal growth, %[1]					
Enterobacter H222	43.6	±	12.3 c	30.5	±	0.8 c
Enterobacter H232	23.8	±	4.3 ab	23.2	±	1.4 bc
Kosakonia H182	24.9	±	6.1 ab	10.0	±	8.1 a
Kosakonia H191	34.9	±	3.9 bc	23.7	±	3.8 bc
Pantoea H186	20.2	±	3.2 a	20.2	±	2.1 b
Leuconostoc H255	20.3	±	2.5 a	20.3	±	2.8 b
Leuconostoc H270	21.7	±	1.2 ab	19.3	±	3.0 b
AvoGreen	33.2	±	3.0 abc	30.4	±	3.3 c

[1]mean ± standard deviation.

Symptom diameter observed after 10 days was slightly lower for bacteria treated spots, especially with isolate H222 (21.1 mm ± 0.9 mm for H222 versus 23.4 mm ± 1.3 mm for the control condition), but the significance was low (p-value = 0.141) (**Figure 3**). Combination of the two isolates did not enhance the observed effect.

Conditions applied before addition of bacteria, *i.e.* wounding of mature fruit and incubation with *C. gloeosporioides* for 24 h, are drastic as they strongly favour fungal development and activate fruit defence mechanisms.

Moreover, the influence of temperature is a crucial parameter for microbiome, as well as the maturity level of mango [85]. *Enterobacter* H222 was isolated at 12˚C but its optimal growth temperature is 27˚C. *L. mesenteroides* can also grow at low temperatures [86] [87] and optimal growth temperature of isolate H255 is 30˚C. It is thus likely that a different effect would have been observed with less mature fruit stored at 12˚C.

Eventually, a pre-harvest application of fruit or application on unripe fruit and optimization of the bacterial population to spray on fruit surface should be considered. A more accurate identification and a careful examination of H222 isolate should be performed prior to consider any further development of biocontrol product in order to assess the safety of the use.

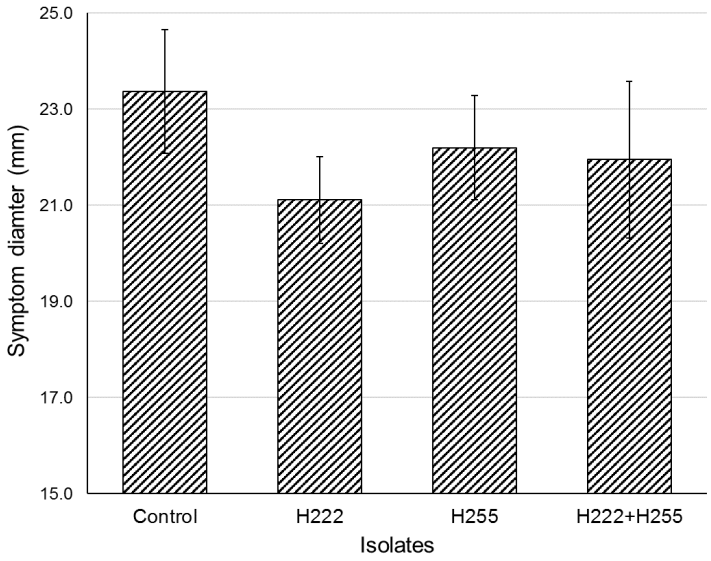

Figure 3. Severity of anthracnose caused by *C. gloeosporioides* MUCL 43868 in wounded cv. José mangoes treated with H222 [*E. cloacae* complex], H255 [*L. mesenteroides*], and H222 + H255 [*E. cloacae* complex and *L. mesenteroides*] and stored 10 days at 20˚C. Severity is expressed as diameter of symptom (mm) on mango.

4. Conclusion

Isolation of 305 epiphytic bacteria from mango surface and identification of 26 isolates showed the presence of species previously observed on other fruit or leaves. Moreover, isolation of *Kosakonia* from carposphere was not previously described.

Fruit post-harvest biocontrol efficacy relies on the ability of biocontrol strains to survive, develop, colonize and exhibit an anti-fungal activity on fruit surface. The relationship between the efficacy and storage temperature should be more deeply investigated, as well as are combination of treatments, in order to increase *in vivo* efficacy. The safe use of isolates has to be particularly watched out.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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