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Efficacy of Microbial Biopesticide Formulations in the control of *Xanthomonas citri* pv. *Mangiferaeindicae* in Cashew (*Anacardium occidentale* L.) in Cote D'ivoire

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Abstract

The cashew tree (*Anacardium occidentale* L.) occupies an important place in the world because of its cashew nut. However, its cultivation is confronted with bacteriosis, a bacterial disease caused by *Xanthomonas citri* pv. *Mangiferaeindicae*. This disease is one of the main causes of the low yield per hectare of cashew nuts, which fluctuates between 350 and 500 kg/ha. In view of this, it is wise to find ways of controlling this disease. It is in this context the objective of this work was to produce bio-formulations based on bacteria isolated from the rhizosphere of cashew trees, in order to evaluate their effectiveness on the growth of the agent responsible for cashew bacteriosis (*Xanthomonas citri* pv. *Mangiferaeindicae*). Thus, two liquid formulations were made from *Pseudomonas fluorescens* and *Bacillus subtilis* isolated from the rhizosphere of cashew. Stability, *in vitro* antagonism and biocontrol tests against *Xanthomonas citri* pv. *Mangiferaeindicae* were performed. The results obtained showed an inhibition of the *Xanthomonas citri* pv. *Mangiferaeindicae* bacterium with inhibition zones of 8.13 ± 2.1 and 25.20 ± 3.9 mm in diameter respectively for the products formulated with *Bacillus subtilis* and *Pseudomonas fluorescens*. In biocontrol tests, both formulated products showed their ability to protect cashew plants against bacterial blight with reduction rates of 80.95 ± 2.3 % and 73.80 ± 5.2% for the *Pseudomonas fluorescens* and *Bacillus subtilis* formulations, respectively. These two formulations of bacterial, once tested in cashew plantations, could be used in the biological control of cashew bacterial blight in Côte d'Ivoire.

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Introduction

Food security is defined as access to safe and sufficient food for all. Meeting the food demand of a rapidly growing world population is becoming a major challenge for humanity. To meet the food needs of the population, agricultural productivity will have to be increased in a sustainable manner worldwide (Kumar *et al*., 2012). However, insect pests and plant pathogens (fungi, bacteria or viruses) contribute to the decline in agricultural productivity, which can be as high as 70%. Indeed, plants as well as harvested and stored products are subjected to attacks by many pathogens (Popp *et al*., 2013). This is the case for cashew (*Anacardium occidentale* L.) in Côte d'Ivoire.Cashew, a crop that plays an important role in the Ivorian economy because of its cashew nut, is a particular strategic and income-generating resource for farmers in the North, South, Centre and East of the country (Soro, 2012). However, despite the economic and nutritional importance of cashew, its cultivation is subjected to several phytopathological problems that compromise the quality and quantity of cashew yield (Silué *et al*., 2017). Bacterial blight is a bacterial disease of cashew caused by *Xanthomonas citri* pv. *Mangiferaeindicae*. This disease manifests itself by oily angular spots on the leaves surrounded or not by a halo-chlorotic. It attacks all the vital organs of the plant with high severity (Zombre *et al*., 2017). In Benin, a work of Afouda *et al.* (2013) revealed average severities of 32.96%. This high severity of bacterial blight could lead to a decrease in cashew nut yield. Also, Soro *et al.* (2017) found evidence of bacterial blight in cashew orchards in Côte d'Ivoire with relative incidences of 15%. To control this disease, producers resort to the use of chemical pesticides (Camara *et al*., 2015).

This strategy can be effective, but the repeated use of these chemicals generates harmful consequences for the environment and the health of the user. Indeed, these products favour the resistance mechanism in pathogens and the ecological imbalance due to the broad spectrum of action of most synthetic compounds. This would lead to the destruction of pests, but also of other populations in the ecosystem and can also cause serious health problems due to pesticide residues in foodstuffs (Kouassi, 2012). In order to mitigate the adverse effects of chemical pesticides, biological control agents are emerging as promising alternatives for the management of crop pathogens. Among these biological agents, microbial biopesticides (bacteria, fungi, viruses) are the most appropriate. Indeed, they offer advantages of higher selectivity and lower toxicity compared to conventional chemical pesticides (MacGregor *et al*., 2006). Recent studies have shown their importance in disease biocontrol (Pérez-Garcia *et al*., 2011). However, the formulation of microbial biopesticides is a key element in the design of control strategies for plant and crop diseases caused by plant pathogens (Nam *et al*., 2018).

During this decade, numerous works in greenhouse and field trials have shown the potential value of rhizosphere bacteria, including *Pseudomonas fluorescens* and *Bacillus subtilis* as biological control agents for plant pathogens (Akram, 2008). A work of Koua (2020) showed that *B. subtilis* strains isolated from the rhizosphere of cocoa trees in Côte d'Ivoire would be effective bioinoculants in the control of cocoa diseases in greenhouses such as swollen shoot. It would therefore be interesting to find a stable bacterial biopesticide formulation suitable for the control of bacterial diseases of cashew trees and thus find a sustainable solution to the problem posed by synthetic products in Côte d'Ivoire. The general objective of this work is to evaluate the efficacy of a formulation of bacterial biocontrol agents based on bacteria (*P. fluorescens* and *Bacillus subtilis*) isolated from the rhizosphere of cashew trees against *Xanthomonas citri* pv. *Mangiferaeindicae.*

Materials and methods

Material

The material used in this study consisted of two bacterial strains isolated from the rhizosphere of cashew (*Pseudomonas fluorescens* and *Bacillus subtilis*) used for the formulation. On the other hand, pathogenic isolates of *Xanthomonas citri* pv. *Mangiferaeindicae* isolated from cashew in Côte d'Ivoire were used to evaluate the effectiveness of the formulations. Greenhouse cashew plants were also used for the biocontrol test.

Methods

Formulation of bacterial biopesticide

Performing pre-cultures

The preculture was carried out by preparing 100mL of YPG (Yeast extract-peptone-glucose) medium distributed in sterile Erlenmeyer flasks at a volume of 50mL per flask. These different media were then each inoculated with a colony of the two bacterial biocontrol agents (*Pseudomonas fluorescens* and *Bacillus subtilis*) previously isolated on solid medium. The pre-culture was then incubated at 30°C for 8 h under agitation at 155 rpm. This preculture was used to inoculate 200mL of bacterial culture.

Bacterial culture preparation

The bacterial culture was carried out in 300mL sterile bottles containing 200mL of YPG (Yeast extract-peptone-glucose). These media were respectively inoculated with 50mL of pre-culture of each bacterial biocontrol agent. These media were then shaken at 155 rpm at 30°C for 72 h. Dissolved oxygen was set at 30% and air flow was adjusted to between 2 and 2.5 L. After 72 h, the resulting bacterial culture was centrifuged at 6000 rpm for 10 min using a refrigerated centrifuge (ACM-CFG-54251, India). The supernatant was collected in 300mL sterile containers and stored at 4°C. To this supernatant was added formulation adjuvants for obtaining bacterial biological product in liquid form (Cabrefiga *et al*., 2014).

Formulation

The formulation was carried out according to the modified Mensah *et al.* (2017) method. Thus, the different formulations were prepared by mixing bacterial supernatant with formulation adjuvants. The adjuvants consisted of 5% glucose protectant (Dextrose, alpha-D(+)-glucose, France), 5% glycerol (Bright, China), 0,8% dispersing agent (Vegetable oil, Extra virgin olive oil, Algeria) and 5% emulsifying agent (Tween® 20 (Polysorbate), Panreac). Subsequently, the mixture of adjuvant and bacterial supernatant obtained was homogenised using a vortex (Velp France brand) for 20 min and then stored in sterile jars, wrapped with aluminium foil and kept at 4°C for the determination of stability and verification of *in vitro* control and biocontrol in the greenhouse.

Stability testing of formulations

The stability of the different formulations was evaluated through a natural ageing test (phase separation observation). For this purpose, the formulations were poured into transparent glasses and left at room temperature (25°C) for observation for up to 30 days. These observations were related to the change in colouring of the solutions over time under the effect of oxidation and water activity without heat treatment of the formulations (Mensah *et al.*, 2017).

In vitro antibacterial test

The bactericidal activity of the bacterial formulations was evaluated *in vitro* against the cashew pathogen *Xanthomonas citri* pv. *Mangiferaeindicae* in Côte d'Ivoire, using the agar diffusion method described by Toty *et al.* (2013). Thus, two media were prepared namely YPGA 100% and YPGA 75% agar media. The YPGA 75% agar medium was obtained by the calculation ratio based on the composition of the 100% YPGA medium. Then, different concentrations (0.1 ; 0.25 ; 0.5 and 1%) of the formulations were prepared in 20mL sterile screw tubes.

These concentrations were obtained by mixing the biocontrol agent formulation with sterile distilled water. Also, a 5mL bacterial suspension at 10^8 CFU/mL was previously prepared from a pure culture of *Xanthomonas citri* pv. *Mangiferaeindicae* aged 24 h. The resulting bacterial suspension was then mixed with YPGA medium (75%) and distributed evenly over the surface of YPGA agar (100%) in Petri dishes. After solidification of the mixture, wells were made using a sterile Pasteur pipette. Subsequently, 50 µL of each concentration of each of the bacterial formulations was placed in each well.

The negative control was inoculated with 50 µL of sterile distilled water. The inoculations were carried out in triplicate. After diffusion of the inoculates into the agar, the cultures were incubated at 30°C for 72 h. At the end of the incubation, the diameters of the *Xanthomonas citri* pv. *Mangiferaeindicae* inhibition zones, which were indicated by a clear zone around the well, were measured with a graduated ruler.

Biocontrol in greenhouses

A suspension of *Xanthomonas citri* pv. *Mangiferaeindicae* (causal agent of cashew bacterial blight) was previously prepared from pure culture aged 24-48 h in 5mL of YPG broth for 24 h. This suspension was used to perform infiltrations between the secondary veins of young cashew nursery leaves using a needleless syringe. This suspension was used to perform infiltrations between the secondary veins of young leaves of cashew tree nurseries using a needle-less syringe. Twenty-four (24) hours after infiltration of the plants with the bacterial suspension, the infected plants were treated with the two formulations based on *Pseudomonas* fluorescens and *Bacillus subtilis*. Five (5) days after application of the different formulations, their capacity to reduce infections due to the pathogenic bacteria was evaluated. This was done by determining the incidence and the disease severity index according to formulae 1 and 2 below. The severity of the disease was assessed using a visual rating scale ranging from 0 to 9. The scale as follows (Groth *et al.*, 1999; Cardoso *et al.*, 2004): 0 = No symptoms; 1 = 1-5%; 3 = 6-10%; 5 = 11-25%; 7 = 26-50%; 9 > 50% of leaf area infected.

$$I\ (\%) = \frac{\text{Number of diseased plants}}{\text{Total number of plants observed}} \times 100 \qquad (1)$$

$$IS = (\sum (Xi \times ni) / N\ Z) \times 100 \qquad (2)$$

- I : Incidence of the disease
- Is : Severity index
- Xi : Note i of the disease ;
- ni : Number of plants with grade i ;
- N : Total number of plants assessed ;
- Z : Highest score.

Determination of the reduction rate of both diseases after treatment

The rate of reduction of bacterial disease by formulations of bacterial biocontrol agents is determined by the following formula :

$$R = (IsT - IsE) / IsT) \times 100 \qquad (3)$$

R : Disease reduction rate

IsT : Severity index of pathogen-infected plants not treated with bacterial biocontrol formulation

IsE : Severity index of pathogen-infected plants treated with biocontrol formulation

Statistical analysis

The collected data were recorded with Excel 2016 spreadsheet and analysed with Statistica version 7.1 software. Analyses of variance (ANOVA) were performed on the mean susceptibility score of *Xanthomonas citri* pv. *Mangiferaeindicae* in the presence of bacterial biopesticide formulations. The normality of the residuals and the homogeneity of the variances were checked. Comparisons between means were made using the Newman-Keuls test at the 5% level.

Results

Duration of stability of formulated biocontrol agents

The natural ageing analysis of the different liquid formulations of bacterial biopesticide showed products with a stability of at least 3 months. Indeed, during the three months of exposure of the formulated products, no phase separation was observed, which indicates the homogeneity of the formulated products. Also, no biological degradation was observed due to the high water activity in these formulated products (Fig. 1).

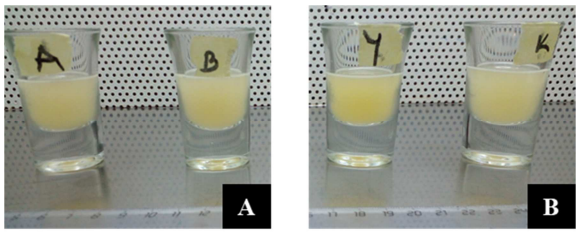

Fig. 1. Stability of different formulations of bacterial biocontrol agents. **A**- Day 0 after formulation et **B**- 3 months after formulation.

In vitro antibacterial activities

The biological efficacy of the different bacterial biopesticide formulations was verified by *in vitro* antibacterial tests against *Xanthomonas citri* pv. *Mangiferaeindicae*, the causal agent of the cashew bacterial disease in Côte d'Ivoire. The results obtained showed an inhibition of the bacterium which is reflected by the appearance of clear and smooth inhibition zones in the wells after the addition of the different formulations (Fig. 2). The diameters of these inhibition zones were 8.13 ± 2.1 and 25,20 ± 3,9 mm respectively for the *Bacillus subtilis* and *Pseudomonas fluorescens* formulations (Fig. 3).

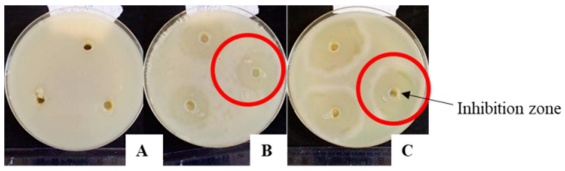

Fig. 2. Inhibition of *Xanthomonas citri* pv. *Mangiferaeindicae* by the formulations : **A** - Control without formulation **B**- *Xanthomonas citri* pv. *Mangiferaeindicae* Vs 1% of *B. Subtilis* formulation and **C-** *Xanthomonas citri* pv. *Mangiferaeindicae* Vs 1% *P. fluorescens* formulation.

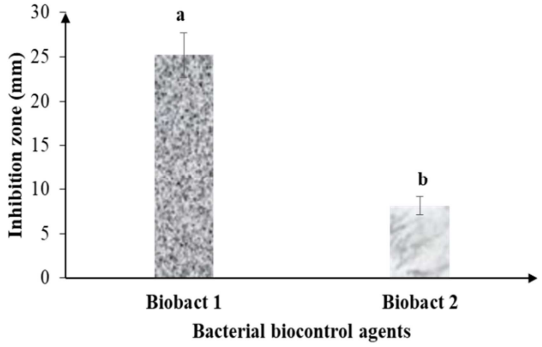

Fig. 3. Effect of different formulations of bacterial biocontrol agents on the growth of *Xanthomonas citri* pv. *Mangiferaeindicae.*

Biobact 1 : *P. fluorescens* formulation and Biobact 2 : *B. Subtilis* formulation

Bands topped by the same alphabetical letter are not statistically different ($p \leq 0.05$) (Newman and Keuls)

In vivo antibacterial activity

Fig. 4 shows the images of the effect of the different formulations against *Xanthomonas citri* pv. *Mangiferaeindicae* on cashew plants. These results show that the different formulations have the ability to confer effective protection to cashew plants. Control plants not inoculated with the pathogen *Xanthomonas citri* pv. *Mangiferaeindicae* and not treated with the bacterial products did not show any symptoms of bacterial blight (Fig. 4 A). Plants inoculated with the bacterial pathogen and not treated with the formulated biological products showed yellowing of the leaves with premature fall of the younger ones (Fig. 4 B). However, plants treated with the formulated bacterial biocontrol agents showed normal growth resulting in normal leaf colouration (Fig. 4 C).

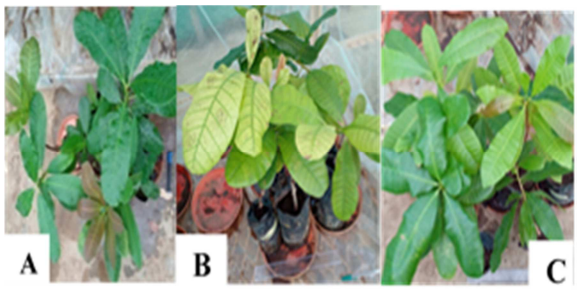

Fig. 4. Development of bacterial blight in treated and untreated plants after one month : **A**- Plants not inoculated with the pathogen and not treated; **B**- Plants inoculated with *Xanthomonas citri* pv. *Mangiferaeindicae* sp; **C**: Plants inoculated with the pathogen and treated with biocontrol agents.

Bacterial blight infection rate after treatment of plants

The results of development of disease occurrence on treated and untreated plants are presented in **Fig. 5**. These results show that all treated and untreated plants showed symptoms of bacterial blight.

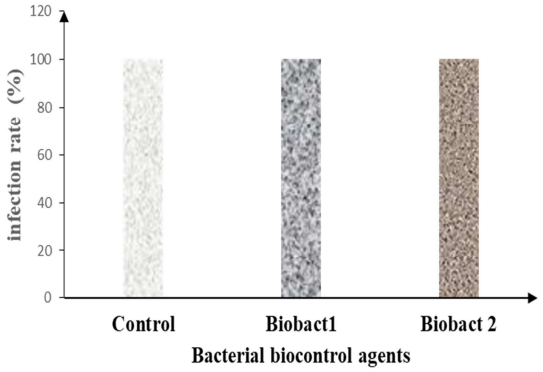

Fig. 5. Development of the of bacterial blight in treated and untreated plants.

Biobact 1 : *P. fluorescens* formulation and Biobact 2: *B. Subtilis* formulation

Average severity of bacterial blight after treatment of plants

Untreated control plants showed an average severity of 28% higher than plants treated with both formulations. Plants treated with the *Pseudomonas* fluorescens formulation had a severity of 5.33% while those treated with *Bacillus* subtilis had a severity of 7.33%. However, statistical analysis showed no significant difference between these two values (Fig. 6).

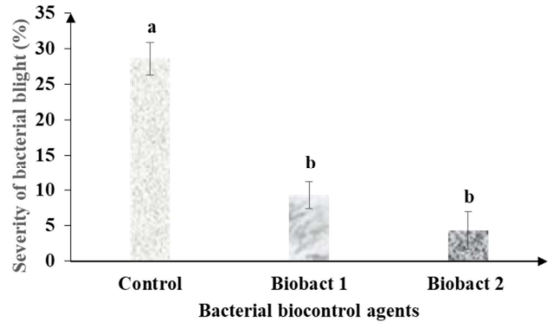

Fig. 6. Average severity of bacterial blight on plants after inoculation with *Xanthomonas citri* pv. *Mangiferaeindicae* and treatment with formulated biocontrol agents.

Biobact 1: *P. fluorescens* formulation and Biobact 2 : *B. Subtilis* formulation

Bands topped by the same alphabetical letter are not statistically different ($p \leq 0.05$) (Newman and Keuls)

Bacterial blight reduction rates by biopesticide formulations

In general, the average reduction rates are above 50% for both formulation types. The values of the reduction rates are 80.95 and 73.80 % for *Pseudomonas fluorescens* and *Bacillus subtilis* respectively. These two values are not statistically different at the 5% threshold (Fig. 7).

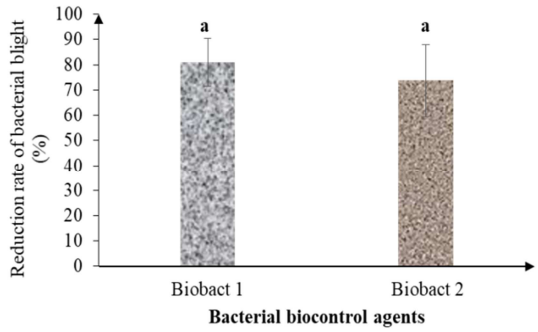

Fig. 7. Reduction rate of bacterial blight on cashew plants by the two formulated biocontrol agents.

Biobact 1 : *P. fluorescens* formulation and Biobact 2 : *B. Subtilis* formulation

Bands topped by the same alphabetical letter are not statistically different ($p \leq 0.05$) (Newman and Keuls)

Discussion

Pathogenic microorganisms (bacteria, fungi, mycoplasma, viruses) and insect pests are generally responsible for 20-40% of pre-harvest crop yield losses (Wojcieh and Lise, 2002). In cashew crops in Côte d'Ivoire, bacterial blight is a bacterial disease that causes huge post-harvest losses. In most cases, this disease is controlled through the use of chemical pesticides. However, these chemical pesticides have harmful effects on the environment and on the health of the user. In view of these disadvantages, it is important to find alternative solutions that will allow continued control of plant pathogens while reducing the use of chemicals. These may involve the development of bacterial biopesticides that would better protect the environment and the user's health (Thakore, 2006). Thus, the general objective of this study was to develop a formulation of bacterial biocontrol agents based on bacteria isolated from the rhizosphere of cashew. The biocontrol agent formulations produced in this study showed good stability over time, lasting up to 3 months. This stability is believed to be due to the presence of formulation adjuvants such as surfactants (Aridity *et al.* 2004; Alvarez-Solano *et al.*, 2006). These results differ from those of Mensah *et al.* (2017) who after formulating biopesticides based on nettle plant (*Urtica dioica* L.) extract found stability lasting seven days.

In vitro antagonism and greenhouse biocontrol tests were carried out against the cashew bacterial blight pathogen *Xanthomonas citri* pv. *Mangiferaeindicae*.. Inhibition of the cashew pathogen by the formulations resulted in zones of inhibition ranging from 8.13 ± 2.1 to 25.20 ± 3.9 mm in diameter. The *Pseudomonas fluorescens* formulation showed the highest antibacterial activity with a diameter of 25.20 ± 3.9 mm. These results are believed to be due to the production of antibacterial substances by these two bacteria involved in the formulation of biological products that would inhibit the growth of *Xanthomonas citri* pv. *Mangiferaeindicae in vitro* (Showkat *et al.*, 2012). This work is in line with that of Nguefack *et al.* (2005) who showed the efficacy of *Ocimum gratissimum* and Thymus vulgaris formulations on the bacterial growth of *Xanthomonas oryzae* pv. oryzicola, the causal agent of leaf streak bacterial disease in rice. In the same vein, the work of Zombré *et al* (2017) on "Antibacterial activity of

extracts of six aromatic plants against *Xanthomonas citri pv. mangiferaeindicae*, bacterium responsible for black spot disease of cashew and mango in Burkina Faso" showed a significant inhibition of *Xanthomonas* bacterium with the plant biopesticide *Ocimum gratissimum*. The inhibition zone diameter observed with this product was 19.85 ± 3.6 mm. This value of inhibition zone diameter is lower than that of the *Pseudomonas fluorescens* supernatant formulation, which is 25.20 ±3.9 mm, but still higher than that obtained with the *Bacillus* sp formulation in our work.

The results of biocontrol tests in the greenhouse with both formulated products showed beneficial effects in protecting cashew plants against the bacterial pathogen. Reduction rates of 80.95% and 73.80% for the *Pseudomonas* fluorescens and *Bacillus subtilis* formulations respectively were observed. These results show that the bacterial strains involved in these formulations would reflect a good possibility of biological control of the cashew plant pathogen. This important reduction rate and the strong growth of the seedlings compared to the controls would be linked to the capacity of these biological agents to produce indole-3-acetic acid and to solubilize phosphorus. Indeed, Fatima *et al* (2009) also showed that the high germination rate and strong growth of the plants were linked to the synthesis of Indole Acetic Acid (IAA). This ability of *B. subtilis* isolates to produce IAA would have enhanced chlorophyll in the leaves. This would explain the fact that the leaves of plants treated with the biopesticide formulations were greener than those treated with the phytopathogenic isolates. Similar results were observed with *Bacillus subtilis* strains BTP1 and BC25 against *B. cinerea* in tomatoes and cucumbers (Akram, 2008).

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