Investigation of the Effectiveness of Three Plant Defense Stimulators on the In Vitro Growth of *Mycosphaerella fijiensis*, Causal Agent of Black Sigatoka Disease of Banana

ABSTRACT

The study aimed at controlling the mycelial growth and germination of *Mycosphaerella fijiensis* in Petri dish by using three plant defense stimulators. Approximately, 2 mL of each stimulator (Vacciplant®, Callel® and calliete®) were added to the different *M. fijiensis* culture media. The results obtained show that Vacciplant® and Callel® promote the in vitro growth of *M. fijiensis* contrary to calliete® which inhibits it. Indeed, it causes total mortality (100%) of conidia at the sporulation stage.

Keywords: *Mycosphaerella fijiensis*, plant defense stimulators, *mycelia, sporulia, pathogen growth*

1. INTRODUCTION

The agricultural world is being confronted with threats of various kinds. In the case of banana production, the most important threats come from fungi, nematodes, bacteria or insects [13]. Foliar diseases caused by the fungus of the genus Mycosphaerella are the most damaging group of diseases to banana [17]. *Mycosphaerella fijiensis*, the fungus responsible for black Sigatoka disease, is considered the most aggressive species of the genus Mycosphaerella associated with banana [4,10]. It is one of the major limiting factors in banana cultivation. Sigatoka disease is the most destructive disease of banana and is present in most production areas between the two tropics [13]. Black Sigatoka disease can cause losses of 50 to 100% under certain conditions [8, 12] as well as possible qualitative depreciation of the fruits [3]. The causal agent of this disease, *Mycosphaerella fijiensis*, is more difficult to control than that of yellow Sigatoka, caused by M. musicola, as reported by Ploetz [17]. Chemical control of the disease involves the intensive use of fungicides, which leads to the development of resistance and human and environmental health problems. For this reason, it is essential to use alternatives to chemical control. Thus, the use of stimulators, which are natural substances that prevent or reduce the damage caused by pathogens, seems necessary. Indeed, these compounds induce different defense mechanisms, thus limiting the risks of resistance of target organisms. In addition, the use of elicitors reduces the use of phytosanitary products and thus delays or avoids the development of pathogen resistance.

To investigate the direct antifungal action or not of the defense stimulators, in vitro tests were performed by confronting a pathogenic source of *M. fijiensis* and the three stimulators (vacciplant®, callel® and calliete®) in a Petri dish.

2. MATERIALS AND METHODS

2.1. Fungi material

The fungal material used in this study is a virulent strain of *M. fijiensis* Morelet ST-W-D98.B1C1. It was isolated from the leaves of a plantain from the CNRA Wanita experimental plantation in Bimbresso (Côte d'Ivoire). This banana is grown in area 98, base 1 and square 1 of this plantation.

2.2. Preparation of culture media

Two types of media (PDA and PDA-V8.) were used in this study. For the preparation of 100 mL of PDA, 2 g of agar, 2 g of D-glucose and 2 g of mashed potato were mixed in 100 mL of distilled water. The mixture was sterilised under a pressure of 1 bar at 120 °C for 30 min in an autoclave. The medium was then poured into Petri dishes under a laminar flow hood. PDA-V8 medium is a PDA medium enriched with a vegetable concentrate called V8. For the preparation of 100 mL, 2 g of agar, 2 g of D-glucose and 2 g of mashed potato were mixed in 80 mL of V8 and the mixture was made up to 100 mL with distilled water. Sterilization was performed as before and then the medium was distributed in Petri dishes under a laminar flow hood.Sterilisation was carried out as before and the medium is dispensed into Petri dishes under a laminar flow hood.

2.3. Inhibition test on mycelium growth of *Mycosphaerella fijiensis*

The mycelium growth inhibition test of *Mycosphaerella fijiensis* was performed with the three stimulators (vacciplant®, callel® and calliete®). For this study, three lots of 10 Petri dishes were made as before and each lot contained PDA medium enriched with 2 mL of the best stimulator or elicitor concentration. Lot 1 was composed of PDA medium + 2 mL of vacciplant® 3%, lot 2 was constituted of PDA + 2 mL of callel® 2% and lot 3 was formed of PDA + 2 mL of calliete® 2%. Agar fragments of about 0.5 cm in diameter containing the fungus were isolated and transferred under hood to Petri dishes with PDA medium supplemented with 2 mL of elicitor. The inoculated Petri dishes were grown for 30 days at room temperature in an incubation room. Controls were made with Petri dishes containing PDA medium without elicitor. The growth of M. fijiensis mycelium grown on elicitor-enriched PDA medium was compared to the control. After the incubation period, the rate of radial mycelial growth inhibition (TRC) relative to the control was calculated according to the formula reported by Camara [2].

$$TRC\ (\%) = \frac{T - E}{T} \times 100$$

T= Average mycelial growth of the fungus (mm) on the control medium ; E= Average mycelial growth of the fungus (mm) in the culture medium at the considered dose of the stimulator.

2.4. Inhibition test on the germination of *Mycosphaerella fijiensis* spores

The study on the inhibition of *Mycosphaerella fijiensis* spore germination was carried out as above. Briefly, three lots of 10 Petri dishes were made and each and each lot contained PDA-V8 medium supplemented with 2 mL of the best stimulator concentration. Therefore, the lot 1 was constiued of PDA-V8 medium + 2 mL of vacciplant® 3%, lot 2 was constituted of PDA-V8 + 2 mL of callel® 2% and lot 3 was formed of PDA-V8 + 2 mL of calliete® 2%.

Petri dishes were then inoculated with 5 mL of the fungal filtrate calibrated at 2.10^5 propagules/mL and incubated in a culture room under continuous light for 14 days to promote sporulation of *M. fijiensis*. Controls were made with Petri dishes containing PDA-V8 medium without elicitor. Sporulation of *M. fijiensis* on elicitor-enriched medium was then compared to that of the control. Thus, 5 mL of sterile distilled water was added to each Petri dish and a spore suspension was prepared by gently scraping the surface of the fungal culture with a curved pasteur pipette. The resulting conidial suspension was filtered through an 80 µm mesh sieve to remove large fragments. A fungal filtrate of 200 µL is harvested and then a few drops of a 2% Trypan blue solution are added according to the Sumantran method [19] in order to stain the vacuoles of the non viable spores and filtered on a Malassez cell [11]. The fungal filtrate is then observed with a DC5.5V/200 mA: LED lamp (white). Non-viable or dead conidia are stained blue in contrast to live cells. This method allows to evaluate the viability of conidia through the germination inhibition rate (GIR) which was calculated according to the following formula:

$$TRG(\%) = \frac{Te - Tt}{Te} \times 100$$

Te = Average number of spores germinated on the culture medium without product ; Tt = Average number of spores germinated on culture media amended with the products

3. RESULTS

3.1. *In vitro* effect of stimulators on the growth of *Mycosphaerella fijiensis* mycelium

Vacciplant®, callel® and calliete® were added to the culture medium of *Mycosphaerella fijiensis* in order to evaluate their effects on mycelium growth (Figure 1). The results showed that the mycelium of *M. fijiensis*, seeded in Petri dishes enriched with vacciplant® is fluffy white, thus alive with a radial growth inhibition rate (TRC) of 0% (Figure 1 A). With callel®, this rate was 20%, i.e. an estimated growth of 80% (figure 1 B). On the other hand, in Petri dishes supplemented with calliete® (D), the rate of inhibition of radial growth was

approximately 99 or even 100% with an isolate that turned brown, thus indicating its death. Calliete® therefore has a fungicidal effect on *M. fijiensis*.

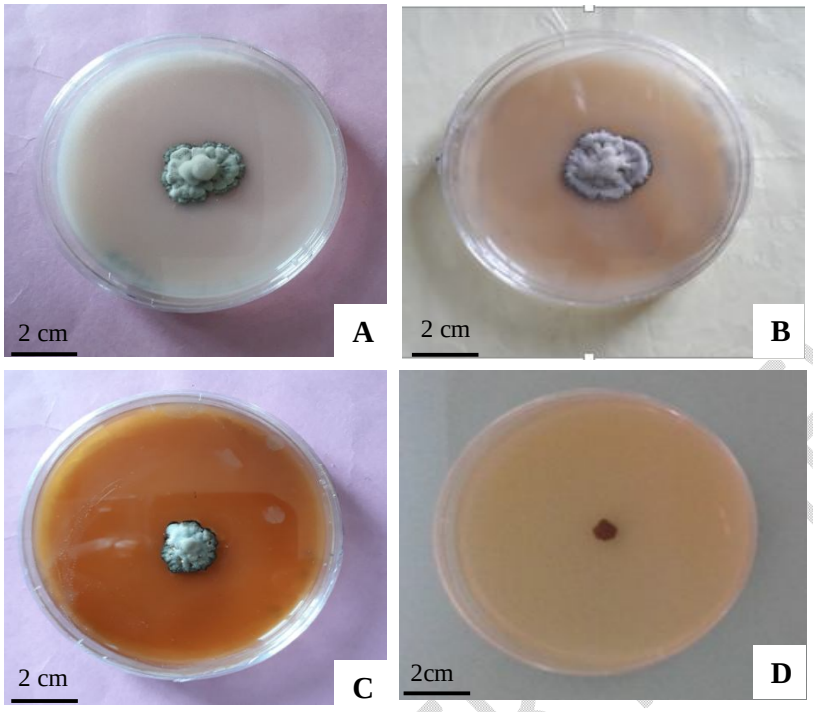

Fig. 1. Macroscopic appearance of the *Mycospharella fijiensis* isolate on the PDA culture medium containing the different stimulators after one month

PDA: Potato Dextrose Agar; A: PDA medium (control); B: PDA + Vacciplant® ;
C: PDA + callel®; D: PDA + calliete® (death of isolate)

3.2. Effect of stimulators on spore germination of *Mycosphaerella fijiensis*

The analysis of figure 2 shows the different levels of sporulation of conidia in different culture media after 14 days of incubation. The results showed optimal development of *M. fijiensis* spores in Petri dishes enriched with vacciplant® or callel® (medium B and C, respectively) as well as in the control medium (medium A), after 14 days in continuous light. Media supplemented with calliete did not induce any sporulation under the same conditions.

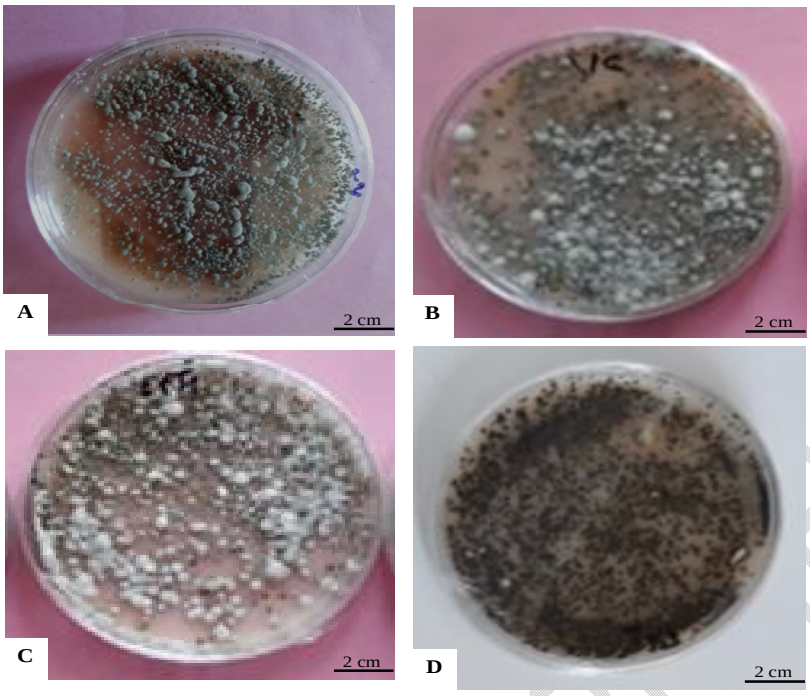

Fig. 2. Macroscopic appearance of the *Mycosphaerella fijiensis* isolate sporulated after 14 days on the PDA culture medium containing the different stimulators

PDA: Potato Dextrose Agar; A : PDA medium (control); B: PDA + Vacciplant®; C: PDA + callel®; D: PDA + calliete® (death of isolate)

Figure 3 shows the evolution of the survival and mortality rate of *M. fijiensis* conidia according to the type of stimulator applied. The analysis of the results did not reveal any significant differences in the germination inhibition rate (TRG) in the media supplemented with vacciplant® or callel®, compared to the control. Thus, this rate was 32.7% for vacciplant® and 34.4% for callel®, i.e. survival rates of 67.3% and 65.5% respectively. On the other hand, in the medium supplemented with calilete®, the rate of inhibition of spore germination was 0%, which translates into 100% mortality.

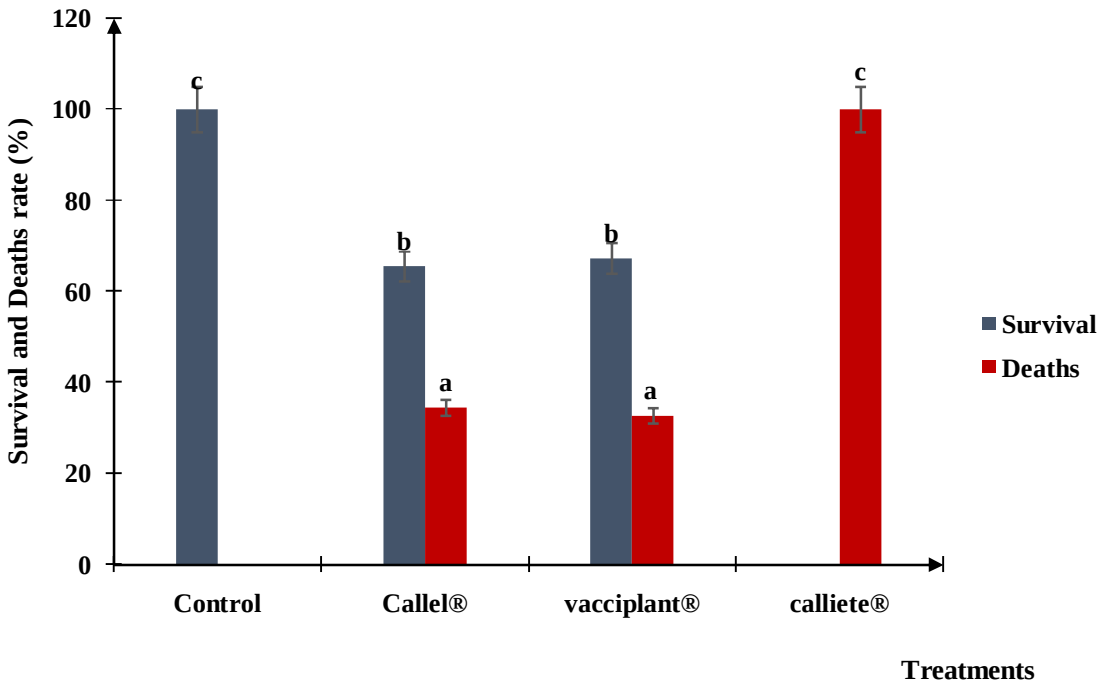

Fig. 3. Evolution of the survival and deaths rates of *Mycosphaerella fijiensis* conidia according to the elicitor treatment

Histograms highlighted with the same letter are not significantly different (Newman-Keuls test at 5%); values represent the mean of triplicate.

4. DISCUSSION

After treatments to reveal the behavior of the fungus Mycosphaerella fijiensis at the mycelial growth stage and at the sporulation stage, media enriched with vacciplant® and callel® allowed fungal growth of the M. fijiensis strain, while media enriched with calliete® inhibited mycelial growth and sporulation. These results suggest that vacciplant and calliete® are not fungicides. In fact, vacciplant®, whose active ingredient is a polysaccharide (Lamarine) derived from the marine alga laminaria digita, promoted growth thanks to its probiotic action, as it is beneficial to the development of microorganisms [20], hence the mycelial and sporuline growth observed. Note also that apart from the basic medium PDA, polysaccharides are a considerable source of energy [7], so the mycelial fragments and spores took advantage of these energy sources to grow. According to ouibrahim [15], polysaccharides have antioxidant activity due to certain enzymes they contain. Thus, the growth of the fungus in the medium containing vacciplant® also seems to be justified by a combined enzymatic action, one from the fungus and the other from the vacciplant®. The development of *M. fijiensis* in the medium enriched with callel® could be explained by the acidification of the culture medium by callel®. In fact, callel® is an ethephon-based elicitor. It is

a plant growth regulator that decomposes into ethylene and phosphoric acid in the presence of water, which gives it an acidity with a pH>4. More over, according to Dix and Webster [6], most pathogenic fungi proliferate in an environment with an optimum growth pH of between 4 and 6. In the case of media enriched with calliete® whose active ingredient is fosetyl-alumium (fosetyl-Al), the death of the fungus was observed. Indeed, this is a pesticide used as a fungicide to control various diseases on crops intended for human and animal consumption [16]. Fosetyl-Al is thought to cause morphological changes in fungal hyphae followed by destruction of the cell walls [5, 9]. This leads to spore lysis and inhibition of mycelial growth of the fungus [21]. Thus, it appears from this observation that calliete® by its direct effect, disrupts the germination of spores and the growth of the mycelium of Mycosphaerella fijiensis in particular through the contribution of aluminium according to Bayer [1]. Moreover, fosetyl-Al, which is a phosphonate, interferes in planta with the metabolism of phosphates in the cells of the pathogen by causing an accumulation of polyphosphates and pyrophosphates [14]. This leads to a disruption of ATP metabolism, i.e. energy metabolism. According to Stehmann and Grant [18], this lack of energy supply to the fungal cells is due to an inhibition of the enzymes of the glycolysis and pentose phosphate pathways; this would lead to an inhibition of the mycelial growth of *M. fijiensis* and even to the death of the pathogen.

5. CONCLUSION

In the context of the search for alternatives to chemical control of the black Sigatoka disease of banana caused by Mycosphaerella fijiensis, a study was conducted to evaluate the direct antifungal effect or not of three compounds used as elicitors. Thus, vacciplant®, callel® and calliete® were added to different culture media of the pathogen in Petri dishes. The results showed that vacciplant® and callel® had no effect on the mycelial growth of M. fijiensis. On the other hand, calliete® inhibited the growth and was even lethal for the pathogen. Thus, only calliete® has a fungicidal effect.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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