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 carried out in this experiment consisted of inhibiting the mycelial growth and germination of the fungus *Mycosphaerella fijiensis* in Petri by using stimulators. For this purpose, 2 mL of each of these three stimulators (Vacciplant®, Callel® and calliete®) were added to the different *M. fijiensis* culture media. The results obtained show that Vacciplant® and Callel® on the contrary promote the growth of *M. fijiensis* in Petri, while calliete® inhibits this growth by influencing a 100% mortality rate of conidia at the sporulation stage.

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

1. INTRODUCTION

The agricultural world is increasingly faced with threats of various kinds. In the case of banana production, the most important threats are from fungi, nematodes, bacteria or insects [13]. Foliar diseases caused by the fungus of the genus Mycosphaerella are the most damaging group of diseases of banana [17]. *Mycosphaerella fijiensis*, the fungus responsible for black Sigatoka disease, is considered the most aggressive species within the *Mycosphaerella* genus associated with banana [4, 10]. It is one of the main limiting factors in banana cultivation. Sigatoka disease is the most destructive disease of banana and is present in most of the production areas between the two tropics [13]. The black Sigatoka disease can cause losses of about 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 is achieved through the extensive use of fungicides, resulting in resistance outbreaks and environmental and human health problems. For this reason, it is essential to use alternatives to chemical control. Thus, the use of stimulators, which are natural substances to prevent or reduce the damage caused by pathogens, seems necessary. Indeed, these compounds induce various defence mechanisms, thus limiting the risks of resistance of target organisms. Furthermore, the use of elicitors allows the reduction of the use of plant protection products and thus delays or avoids the development of pathogen resistance. To verify whether the elicitors used in this study have a direct antifungal action or not, in vitro tests were initiated 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 composed 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 200 µL fungal filtrate was collected and then a few drops of a 2% Trypan blue solution was added according to the method of Sumantran (2011) in order to stain the vacuoles of non-viable spores and filtered on a Malassez haemattimeter [11].

The fungal filtrate is then observed with a DC5.5V/200 mA Lamp : LED (white). The non-viable or dead conidia are colored in blue unlike the living cells. This method allows the viability of conidia to be assessed through the germination inhibition rate (TRG) 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 calliet® 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*.

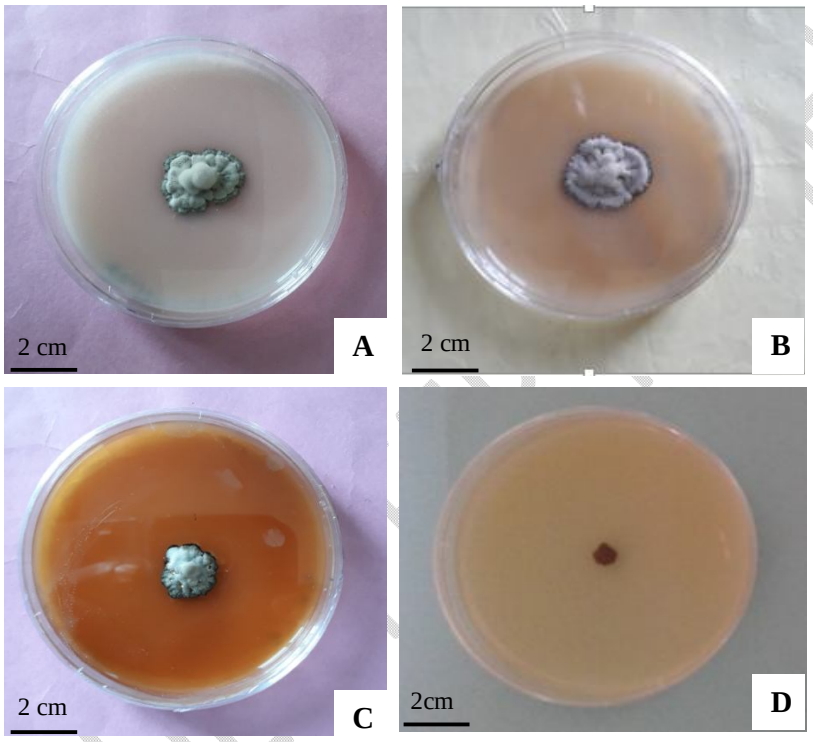

Figure 1: Macroscopic appearance of the *Mycospharella fijiensis* isolate on the PDA culture medium containing the different stimulators after one month

PDA: Potato Dextrose Agar; A: single PDA medium (control); B: vacciplant-containing medium; C: callel-containing medium; D: calliete-containing medium (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 enriched with calliet did not induce any sporulation under the same conditions.

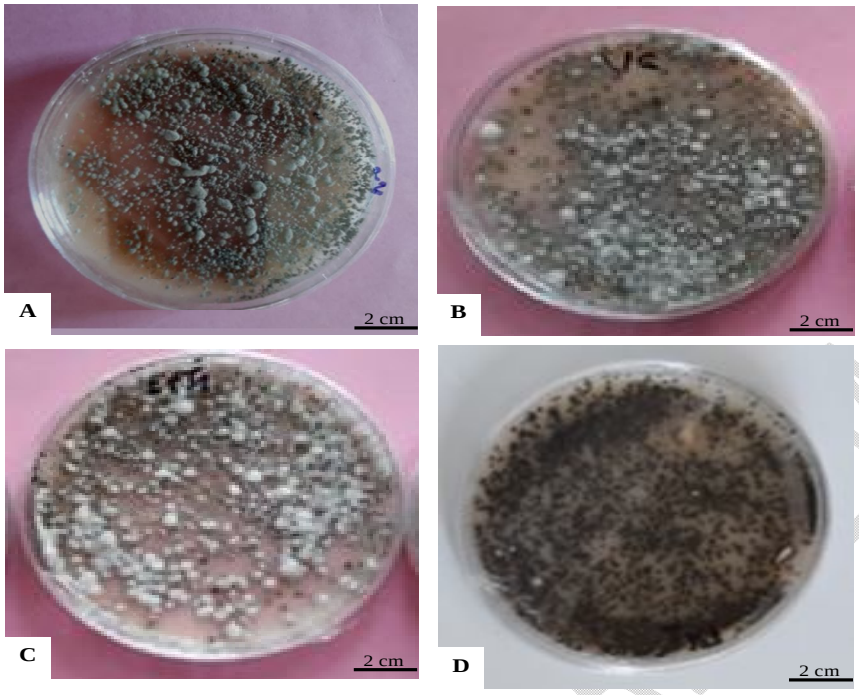

Figure 2: Macroscopic appearance of the *Mycosphaerella fijiensis* isolate sporulated after 14 days on the PDA culture medium containing the different stimulators

PDA: Potato Dextrose Agar; A: single PDA medium (control); B: vacciplant-containing medium; C: callel-containing medium; D: calliete-containing medium (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 enriched with calilete®, the rate of inhibition of spore germination was 0%, which translates into 100% mortality.

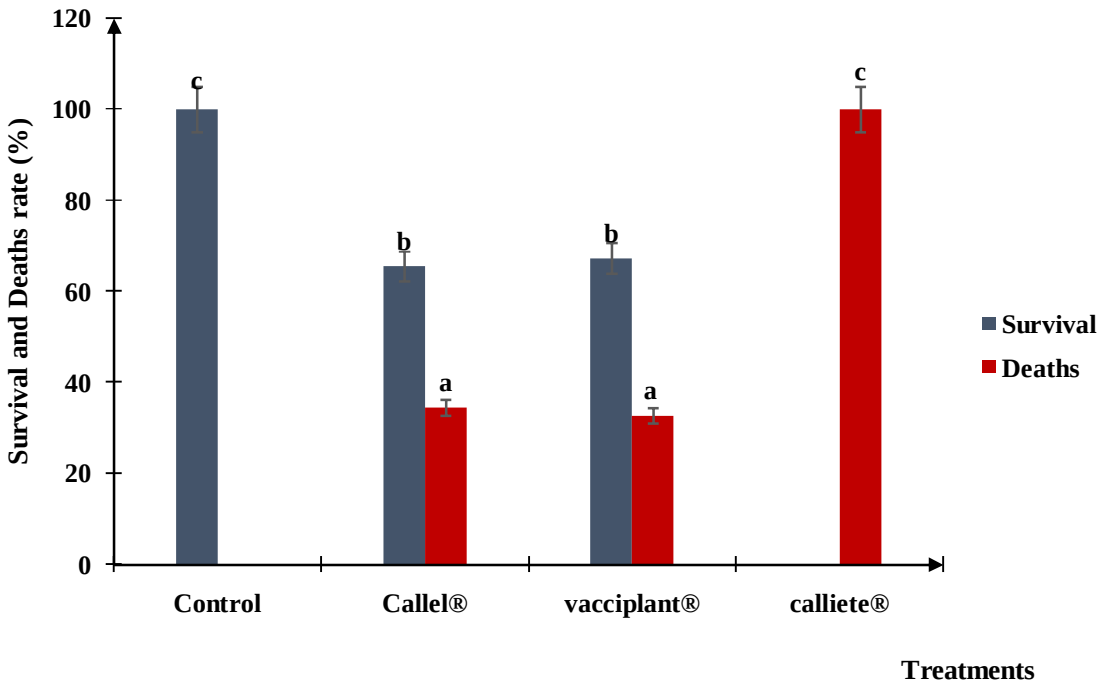

Figure 3 : Evolution of the survival and deaths rates of *Mycosphaerella fijiensis* conidia according to the elicitor treatment

Histograms overlaid with the same letter are not significantly different (Newman-Keuls test at 5%); values represent the average of three replicates.

4. DISCUSSION

Following treatments to reveal the behaviour of the fungus *Mycosphaerella fijiensis* at the mycelial growth stage and at the sporulation stage, the media enriched with vacciplant® and callel® allowed the fungal growth of the *M. fijiensis* strain, where as the media enriched with calliete® inhibited both mycelial and sporulation growth. These results suggest that vacciplant and callel are not fungicides. Indeed, 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. Also, let us note that apart from the basic medium PDA, polysaccharides constitute a considerable energy source [7] so the mycelial fragments and spores took advantage of these energy sources to grow. According to the author ouibrahim [15] polysaccharides have an antioxidant activity thanks to certain enzymes found within them, the growth of the fungus in the medium containing vacciplant also seems to be justified by a combined enzymatic action, one coming from the fungus on the one hand and the other from vacciplant® on the other hand 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]. More over, 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 & 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 framework of the search for alternatives to chemical control of the black Sigatoka disease of banana caused by *Mycosphaerella fijiensis*, a study was carried out 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. From the growth pattern observed, it was concluded that vacciplant® and callel® had no effect on the mycelial growth of *M. fijiensis*, whereas calliete® inhibited this growth and was even lethal to the pathogen. Thus, vacciplant® and callel® have no fungicidal effect on *M. fijiensis* unlike calliete®.

REFERENCES

1. Bayer, 2018. Fosétyl®-Al : premier stimulateur de défense des plantes homologué en vigne. https://www.bayer-agri.fr/cultures/fosetylr-al-premier-stimulateur-de-defense-des-plantes-homologue-en-vigne_4683. Accessed 11/03/2022.

2. Camara B., Dick E., Sako A., Kone D., Kanko C., Boye M.A.D., Ake S Anno A., (2010). Lutte biologique contre *Deightoniella torulosa* (Syd) Ellis, par l'application des

huiles essentielles *d'Eucalyptus platylla* F. Muell. et de *Melaleuca quinquenervia* L. Phytothérapie, 8 :240-244.

3. Castelan F.P, Saraiva L.A et De Bellaire L.L, (2012). Effects of black leaf streak disease and sigatoka disease on fruit quality and maturation process of bananas produced in the subtropical conditions of southern Brazil. Crop Protection, 35 : 127-328.
4. Churchill A.C.L. (2011). *Mycosphaerella fijiensis*, the black leaf streak pathogen of banana: progress towards understanding pathogenbiology and detection, disease development, and the challenges of control. Molecular Plant Pathology, 12: 307-328.
5. Daniel R & Guest D, (2005). Defence responses induced by potassium phosphonate in *Phytophthora palmivora*-challenged *Arabidopsis thaliana*. Physiological and Molecular plant pathology. 67:194-201.
6. Dix N. J. & Webster J. (1995**).** Fungi of Extreme Environments. n: Fungal Ecology, Springer, Dordrecht, pp. 322-340.
7. Djafar S.M.I., (2018). Les activités biologiques des polysaccharides. Mémoire de Master, Faculté des Sciences de la Nature et de la Vie, Université des frères Mentouri Constantine (Algérie)
8. El Hadrami A. (2000). Caractérisation de la résistance partielle des bananiers à la maladie des raies noires et évaluation de la variabilité de l'agressivité de l'agent causal Mycosphaerella fijiensis. Thèse de Doctorat en Sciences Agronomiques, Faculté Universitaire des Sciences Agronomiques de Gembloux, Belgique, 153 p.
9. Gomèz-Merino F. C. & Trejo-Téllez L. I. (2015). Biostimulant activity of phosphite in horticulture. Scientia Horticulturae. 196:82-90.
10. Koné D. (2008). Contribution à l'étude des champignons epiphylles des bananiers en Côte d'Ivoire: Caréctéristion morphologique et pathologique de *Mycosphaerella fijiensi*s var. difformis *Morelet, Cladosporium musae Mason, Deightoniella turulosa (Syd.) Ellis et Cordana* musae Zim. ; Sensibilité à quelques fongicides. Thèse de Doctorat d'Etat. Laboratoire de Physiologie Végétale, UFR Biosciences, Université de Cocody-Abidjan, 244 p.
11. Keuete K E, Tsopmbeng N. G. R. & Kuiate J. R. (2016). Cultural and morphological variations of *Colletotrichum* spp associated with anthracnose of various fruits in Cameroon, International Journal of Environment, Agriculture and Biotechnology
12. Lassoudière A. (2007). Le bananier et sa culture. Editons Quae, Paris, France, 383 p.
13. Mourichon X. & Fullerton R. (1990). Geographical distribution of two species *Mycosphaerella musicola* Leach (*Cercospora musae*) and *M. fijiensis* morelet (*Cercospora fijiensis*), respectively agents of Sigatoka and Black leaf streak diseases in bananas and plantains. Fruits, 45: 213-218.

14. Niere J.O., Deangelis G. & Grant B.R. (1994). The effect of phosphonate on the acid-soluble phosphorus components in the genus Phytophthora. *Microbiology,* 140 : 1661–1670.
15. Ouibrahim A, (2015**).** Evaluation de l'effet antimécrobien et antioxidant de trois plantesaromatique (*Laurus nobilis* L, *Ocimum basilicum* L et *Rosmarinus officinlis* L.) de l'Est Algérien. Thèse de l'Université Badji mokhtar- Annaba (Algérie).
16. Phytocontrol, 2019. Le fosétyl d'aluminium et l'acide phosphorique. https://www.phytocontrol.com/actualites/le-fosetyl-aluminium-et-lacide-phosphonique. Accessed 11/03/2022.
17. Ploetz R. C. & Pegg K. G. (2000). Fusarium wilt. In Diseases of Banana, Abacá and Enset (D.R. Jones, ed.). CABI Publishing. Wallingford, UK, pp. 143-159.
18. Stehmann C & Grant B. R. (2000). Inhibition of enzymes of the glycolytic pathway and hexose monophosphate bypass by phosphonate. Pesticide Biochemistry and Physiology, 67: 13-24.
19. Sumantra V. N., Joshi A.K., Boddul S., Koppikar S.J., Warude D., Patwardhan B., Chopra A., Chandwaskar R. & Wagh U. V. (2011). Antiarthritic Activity of a Standardized, Multiherbal, Ayurvedic Formulation containing *Boswellia serrata*: *In Vitro* Studies on Knee Cartilage from Osteoarthritis Patients. Phytotherapy Research, 24(5):1375-1380.
20. Wang X., Huang M., Yang F., Sun H., Zhou H., Guo Y., Wang X. & Zhang M. (2015). Rapeseed polysaccharides as prebiotics on growth and acidifying activity of probiotics in vitro. Carbohydrate Polymers, 125:232-240.
21. Wong M. H., 2006**.** Phosphite induces morphological and molecular changes in Phytophthora species. https://researchrepository.murdoch.edu.au/view/author/Wong, Mee-Hua.html. Accessed 11/03/2022.