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Metarhizium anisopliae against *Prosoestus* Spp., Pests of Female Oil Palm Inflorescences: Preliminary Laboratory Tests

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Abstract: *Prosoestus sculptilis* and *Prosoestus minor* (Coleoptera, Curculionidae) are female inflorescences of the oil palm pest. These insects can cause a considerable decrease (about 40%) of the setting rate. The currently proposed control method is the chemical control with the use of thiocyclam-hydrogen-oxalate, derived from the Nereistoxin. For sustainable agriculture, it is appropriate to use an integrated pest management system, including biological control. The purpose of this work is to contribute to the biological control of *Prosoestus* spp. by the use of *Metarhizium anisopliae*, entomopathogenic fungus, to improve fruit set rate of oil palm. A screening of five (05) isolates of the fungus was performed by passive and active suspensions inoculation of spores in increasing concentrations. Coded isolates CNRA-BME2 and CNRA-BME5 were most virulent for these insects with mortality rates of approximately 80% during the passive inoculation on fungic cultures. The concentration c = 10^9 spores/mL was optimal during active inoculation. *Metarhizium anisopliae* is therefore a potential biological control agent against *Prosoestus*.

Key words: Biological control, *Prosoestus* spp., oil palm tree, fruit set rate.

1. Introduction

The cultivated oil palm *Elaeis guineensis* Jacquin (1763) is native to the humid intertropics zone of Africa. Palm cultivation has been booming for about twenty years. This palm tree is used to produce palm oil and palm kernel oil, respectively extracted from the pulp and almond of the fruit [1, 2]. With a production of 60 million tons (Mt) of oil a year (54 Mt palm and 6 Mt palm kernel), the oil palm is the world's first oil plant and a strategic crop for many tropical countries [3]. However, the unmet need for fats remains high, global demand is growing by 3% per year [3]. This deficit is estimated at about 500,000 t in the WAEMU* (West African Economic and Monetary Union) area and just over 1,800,000 t in ECOWAS* (The Economic Community of West African States). Côte d'Ivoire has set a goal of increasing its production from 400,000 tons of crude palm oil to 600,000 tons in 2020 [4]. However, this issue may be compromised by attacks of various pests and diseases [5-7]. Indeed, all parts of the oil palm are exposed to multiple order pests. At the level of female inflorescences, the main pests are *Prosoestus sculptilis* FAUST and *Prosoestus minor* MSHL (Coleoptera, Curculionidae) [8]. These insects live and breed on the female flowers. They feed on stigmas, which can lead, in case of strong aggression, to a drop of more than 40% in the fruit set rate [8]. The only control currently proposed against these pests is chemical control by the use of Evisect S (thiocyclam-hydrogen-oxalate). In a current context of sustainable agriculture and environment preservation, we are turning more and more towards biological

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control by reducing the use of chemicals. The present study was initiated to contribute to the biological control against *Prosoestus* spp. by the use of *Metarhizium anisopliae*, entomopathogenic fungus, to improve the fruit set rate of oil palm fruit in Côte d'Ivoire.

2. Materials and Methods

2.1 Study Site

The work was carried out in Côte d'Ivoire, at the experimental station of La Mé (Latitude: 5°26' North and Longitude: 3°50' West), property of the National Center for Agronomic Research. The natural vegetation of this locality belongs to the ombrophilous sector [9]. The climate is equatorial transition [10], with a relative humidity of 70-80%, the average temperature of about 27 to 28 °C and an annual rainfall of approximately 1,600 to 1,700 mm [7].

2.2 Materials

2.2.1 Plant Material

The oil palm (*Elaeis guineensis*), of *Tenera* type and of the C1001F variety currently popularized in Côte d'Ivoire, was plant material. This variety has a good oil production (4.3 t/ha/year), a low growth rate in height (45 cm/year), a good quality of oil and a tolerance to fusarium. The principal organs of the plant used are female inflorescences and male inflorescences in full anthesis. The plot on which the experiments were conducted is plot J31 (7.9 ha, 10 years old) at La Mé research station.

2.2.2 Animal Material

The animal material consists essentially of *Prosoestus sculptilis* and *Prosoestus minor*, oil palm female inflorescences pests. These insects were collected from the J31 plot of La Me.

2.2.3 Fungal Material

The fungus used is *Metarhizium anisopliae*. The tested *Metarhizium* isolates come from the collection of the Entomology and Phytopathology Laboratory of the National Center for Agronomic Research (Anguededou, Côte d'Ivoire). These are local strains isolated from black banana weevil *Cosmopolites sordidus* in Côte d'Ivoire [11]. These are the CNRA-BME2, CNRA-BME5, CNRA-DME1, CNRA-EGL1 and CNRA-METE (Laboratory Names) coded isolates.

2.3 Methods

Prosoestus sculptilis and *P. minor* were kept in breeding to carry out the different experiments.

2.3.1 Breeding of *P. Sculptilis* and *P. Minor*

Prosoestus sculptilis and *P. minor* were reared in the laboratory in 48 breeding boxes. Ten insects from each of these two species were introduced into 24 boxes. A spikelet of a female inflorescence in full anthesis was put in each of the breeding boxes. These spikelets served as a refuge for insects and the flowers they carry constitute their food. During breeding the spikelets were replaced every two days in different boxes.

2.3.2 Screening of *Metarhizium* Isolates by Insect Inoculation on Fungal Colonies

These inoculations consisted in contaminating the insects by letting them walk for 3 minutes in 11 cm diameter Petri dishes containing 15-day fungal colonies. Ten insects of the same species are inoculated with one of the isolates before being transferred to a breeding box. For each isolate 4 repetitions were carried out, so 40 insects inoculated per species of *Prosoestus*. For each species, 40 control individuals were directly reared in 4 breeding boxes, at a rate of 10 insects per box, without being in contact with the fungus.

The insects are observed every day and the dead are removed and counted. Dead insects are individually disinfected with sodium hypochlorite (1%) for 30 seconds and rinsed 3 times with sterile distilled water. The insect thus disinfected is then placed on filter paper impregnated with distilled water in sterile Petri dishes and incubated in the dark. Time to onset of mycelial down is noted. The fungus is then identified by light microscopy. The mortality rate of both species according to isolate *Metarhizium* applied was followed

for two weeks. Mortality of control insects that were not inoculated was also monitored during this same period.

2.3.3 Inoculation of *Prosoestus* spp. by Spraying Suspensions of Spores at Increasing Concentrations in the Laboratory

This inoculation was done by spraying 10 mL of a spore suspension of 10 live weevils on an inflorescence spikelet in rearing medium with a sprinter.

Effective selected isolates for inoculation of the fungal colonies (BME2 and BME5) were cultured for 21 days PDA (Potato Dextrose Agar) environments. The mycelium formed in the culture dishes was scraped with a sterile spatula and stirred at 150 rpm for 30 minutes using an electric stirrer. The solution obtained was filtered twice on muslin and the concentration of the spore suspension was determined at the Malassez hematimeter (Prof = 1/5m/m).

The concentrations were then adjusted to 10^7, 10^8, 10^9 and 10^{10} spores/mL. Two drops of an aqueous solution of 0.2% Tween-80 were added to 10 mL of spore suspension just prior to spraying to facilitate spore adhesion to the insects. Both isolates were applied to each of the *Prosoestus* species (*P. sculptilis* and *P. minor*) at these four increasing doses. The control insects were treated with a solution of 10 mL of water and two drops of 0.2% Tween-80 aqueous solution. Each isolate was repeated 4 times and the experiment was repeated 2 times. Insects are also observed every day and the dead are removed and counted. Dead insects are individually disinfected with sodium hypochlorite (1%) for 30 seconds and rinsed 3 times with sterile distilled water. The insect thus disinfected is then placed on filter paper impregnated with distilled water in sterile Petri dishes and incubated in the dark. Time to onset of mycelial down is noted. The fungus is then identified by light microscopy. The mortality rate of both species according to isolate *Metarhizium* applied was followed for two weeks. Mortality of control insects that were not inoculated was also monitored during the same period.

2.4 Data Analysis

The Shapiro-Wilk test was applied beforehand to verify the normality of all the variables measured. When the collected data follow a normal distribution, an analysis of variance was performed using the STATISTICA 7.0 software to test the treatment effect on the observed parameter. In the opposite case, the Kruskal-Wallis H test is applied to test the treatment effect on the measured parameter. The averages were compared using the Student-Newman-Keuls test at the 5% threshold.

3. Results

3.1 Screening of Metarhizium Isolates by Inoculating Prosoestus spp. on Fungal Colonies

3.1.1 Mortality Rate of Inoculated Insects

Compared to the untreated control, the different *Metarhizium* isolates tested caused the death of *Prosoestus* spp. *Prosoestus sculptilis* was the most sensitive to the fungus. The BME2 and BME5 isolates were the most effective against these pests.

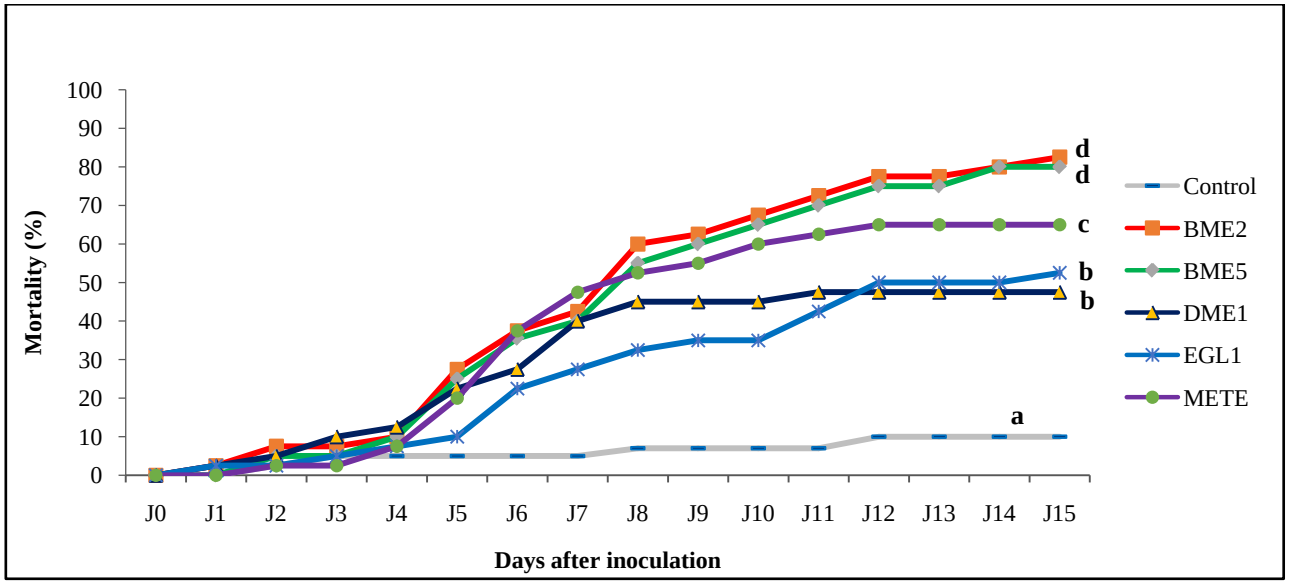
- *Prosoestus sculptilis*

The lethal time 50 (LT50) was 8 days with the BME2 isolates BME5 and METE and 15 days for EGL1. The DME1 isolate did not achieve 50% mortality.

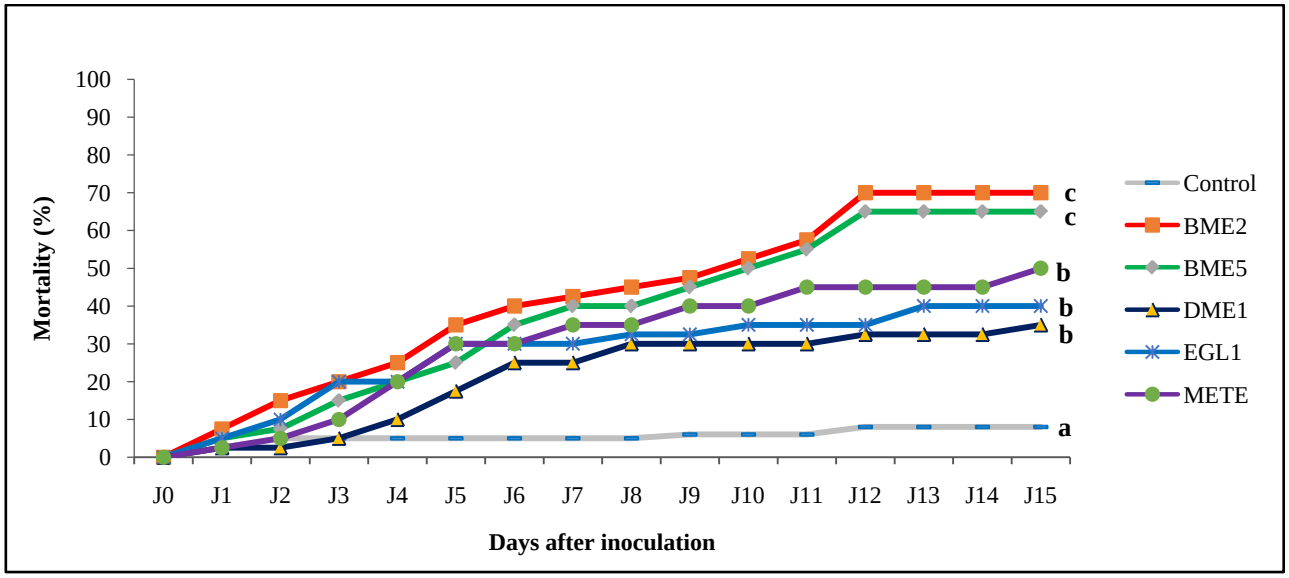
BME2 and BME5 were the most virulent isolates for this pest with respectively 82.5% and 80% cumulative mortality rate at the end of the experiment. The METE isolate resulted in a 65% cumulative mortality rate. The EGL1 and DME1 isolates with respectively 52.5% and 47.5% of the cumulative mortality rate, were the least virulent (Fig. 1a).

- *Prosoestus minor*

The BME2 and BME5 isolates were the most virulent with a 9- to 10-day TL50 and cumulative mortality rates of 70% and 65%, respectively. The other three isolates, METE, EGL1 and DME1 were significantly less virulent for this pest with 50%, 40% and 35% respectively (Fig. 1b).



(a)



(b)

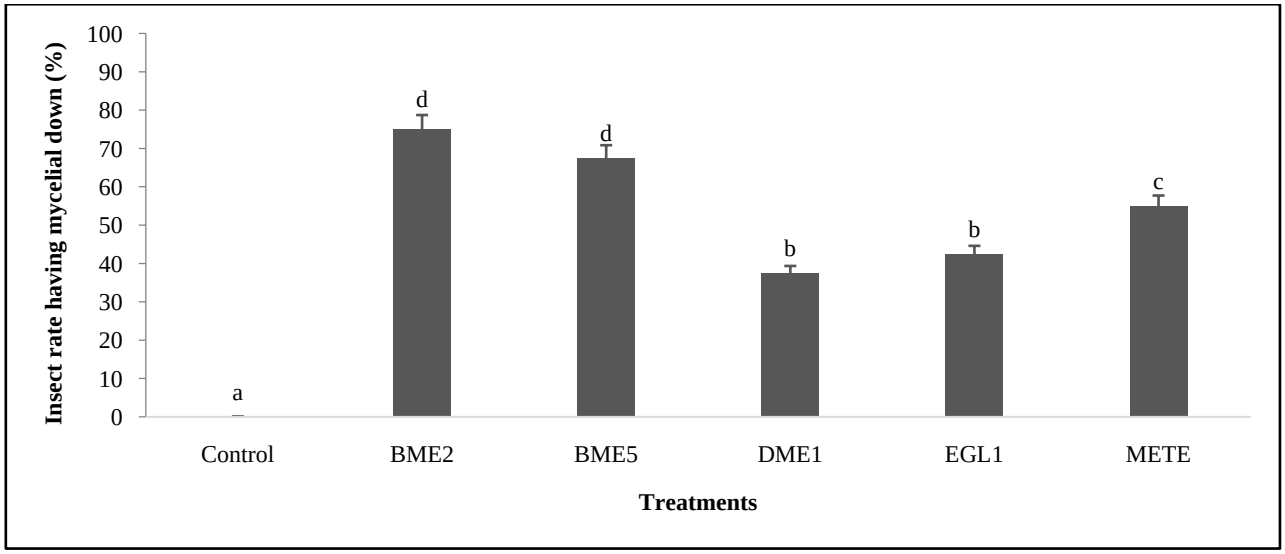
Fig. 1 Cumulative mortality rate of *P. sculptilis* (a) and *P. minor* (b) inoculated on *metarhizium anisopliae* isolates colonies (marked isolates with the same letter belong to the same homogeneous group).

3.1.2 Mortality Rate and Sporulation of the Fungus on Dead Insects

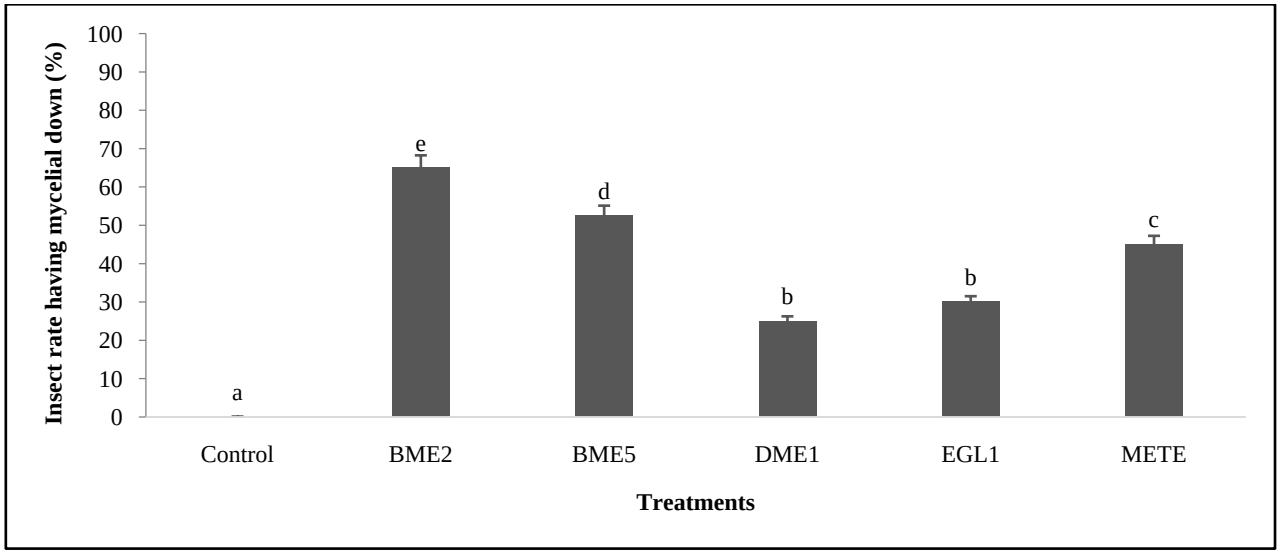
- *Prosoestus sculptilis*

Several insects presented the mycelia fuzz of *Metarhizium* after their death. A significant difference was observed at the 5% level and 4 classes were formed in ascending order of the rate of insects with mycelian *Metarhizium* down. The BME2 and BME5 isolates recorded the highest percentages with downy appearance on respectively 75% and 67.5% of the initial number of insects. With 55% of the initial insect population, the downside of the METE isolate appeared less compared to those of BME2 and BME5, but more than those of EGL1 (42.5%) and DME1 (37.5%). No myelial flush of *Metarhizium* was observed in the control (Fig. 2a).

The appearance of fluff mycelial *P. Sculptilis* death varied from one isolate to another without actually



(a)



(b)

Fig. 2 Rate of dead *P. sculptilis* (a) and *P. minor* (b) with mycelial *metarhizium* down.

Table 1 Characteristics of fungi developed on *Prosoestus* spp. deaths after passive inoculation on colonies of *metarhizium* isolates.

Isolates	*Prosoestus sculptilis*		*Prosoestus minor*	
	Time of onset of mycelium (j)	Sporulation on dead insects ($\times 10^5$ spores/mL)	Time of onset of mycelium (j)	Time of onset of mycelium (j)
BME2	2.3 a	90.66 c	2.25 a	80 c
BME5	2.9 a	60.33b	3.1 a	56.66 b
DME1	2.1 a	97 d	2 a	89.33 d
EGL1	3.1 a	34.66 a	3.35 a	25.66 a
METE	2.65 a	57.66 b	2.9 a	54.33 b
Probability (p)	0.15	< 0.001	0.2	< 0.001
Significance	NS	S	NS	S

NS: Not significant; S: Significant.

having an isolate effect (F = 3.6; $p = 0.15$). This delay was 2.1 to 3.1 days with an average of 2.61 days (Table 1).

- *Prosoestus minor*

The myelial flush of *Metarhizium* was observed on several individuals of *P. minor* after their death. A significant difference was obtained at the 5% level. The BME2 isolate is the one whose down has been observed on the most insects with 65%. It is followed by BME5 isolates with 52.5% and METE (45%). Few insects presented the down of mycelium and egl1 DME1 isolates. These two isolates belong to the same homogeneous group with 30% and 25% respectively. No myelial flush of *Metarhizium* was observed in the control (with 0%); Fig. 2b.

The time to onset of mycelial down on *P. minor* death also varied from one isolate to another without truly having an isolate effect (F = 3.2, $p = 0.2$). This delay was 2 to 3.4 days for an average of 2.72 days (Table 1).

Regarding the number of spores produced on dead weevils after this inoculation, an isolate effect was observed (F = 169.16, $p < 0.001$). The DME1 isolate had the highest sporulation (97×10^5 spores/mL on *P. sculptilis* and 89.33×10^5 spores/mL on *P. minor*). It is followed by the BME2 isolate with 90.66×10^5 spores/mL on *P. sculptilis* and 80×10^5 spores/mL on *P. minor*. BME5 isolates (60.33×10^5 spores/mL on *P. sculptilis* and 56.66×10^5 spores/mL on *P. minor*) and METE (57.66×10^5 spores/mL on *P. sculptilis* and 54.33×10^5 spores/mL on *P. minor*) come next. The EGL1 isolate which gave 34.66×10^5 spores/mL on *P. sculptilis* and 25.66×10^5 spores/mL on *P. minor* was the least sporulating (Table 1).

3.1.3 Partial Conclusion

The five (05) *Metarhizium* isolates tested expressed entomopathogenic potency against *Prososestus sculptilis* and *Prosoestus minor* at different levels. The BME2 and BME5 isolates were the most virulent. They were therefore selected for the inoculation test of *Prosoestus* spp. with increasing doses of spore suspensions.

3.2 Laboratory Efficacy of Metarhizium Isolates after Inoculation of Prosoestus spp. *with Spore Suspension in Increasing Concentrations*

3.2.1 Mortality Rate of *Prosoestus spp*. Depending on Increasing Concentrations of Spores of BME2 and BME5

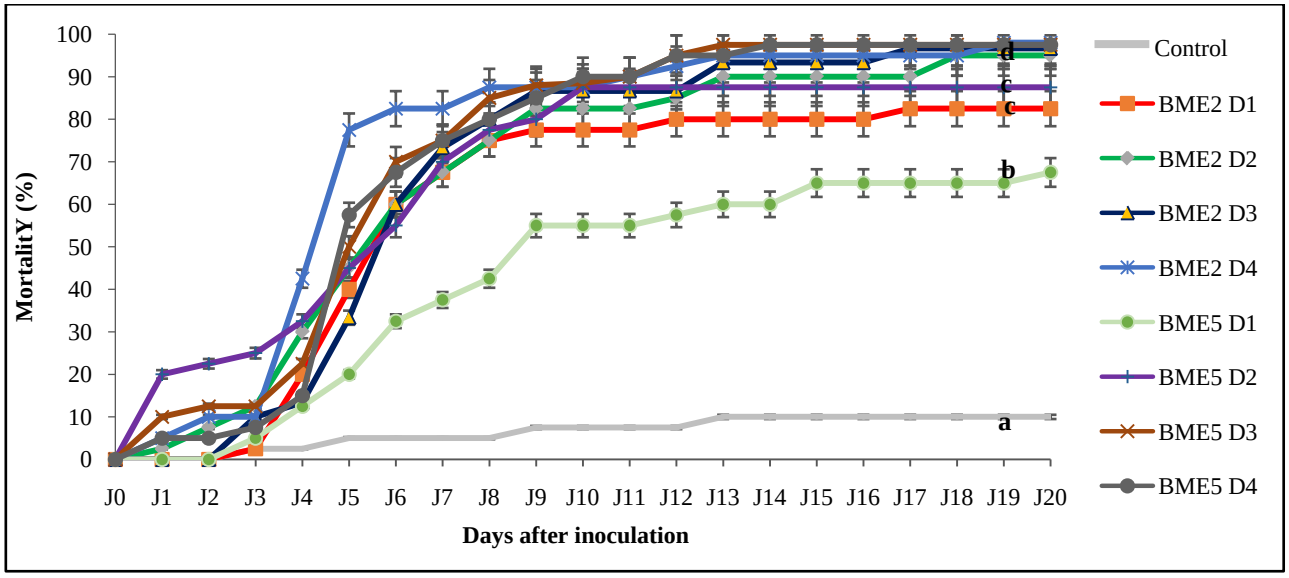
- *Prosoestus sculptilis*

With a lethal time (LT) for 90% of the population from 10 to 11 days, BME5 with $c = 10^{10}$ spores/mL, BME5 with $c = 10^9$ spores/mL and BME2 with $c = 10^{10}$ spores/mL were most virulent for *P. sculptilis*. BME2 $c = 10^9$ spores/mL and BME2 $c = 10^8$ spores/mL, followed with LT90 respectively 12.5 and 13 days (Fig. 3a). BME5 $c = 10^8$ spores/mL and BME2 $c = 10^7$ spores/mL were less virulent compared to previous treatments. These two treatments did not achieve 90% mortality. BME5 $c = 10^7$ spores/mL was the least virulent for *P. sculptilis* with a LT50 of 8.8 days at the end of the experiment (15j) (Fig. 3a). The statistical analysis showed a significant difference at the 5% level and the Student-Newman-Keuls test yielded four distinct classes a, b, c and d in the ascending order of the mortality rate. With mortality rates ranging from 95% to 98%, the concentrations $c = 10^8$ spores/mL, $c = 10^9$ spores/mL and $c = 10^{10}$ spores/mL of the BME2 isolate as well as $c = 10^9$ spores/mL and $c = 10^{10}$ spores/mL of the BME5 isolate were the most virulent for *P. sculptilis* (Fig. 3a). Followed BME5 $c = 10^8$ spores/mL and BME2 $c = 10^7$ spores/mL with 87.5% and 82.5% respectively of cumulative mortality, the lowest concentration of BME5 ($c = 10^7$ spores/mL) was the least virulent for this insect with a cumulative mortality rate of 67.5% (Fig. 3a).

- *Prosoestus minor*

No isolate at the tested concentrations achieved 90% mortality rate after 15 days. The highest concentrations ($c = 10^{10}$ spores/mL and $c = 10^9$ spores/mL) of BME5 gave the shortest LT50, 7 days (Fig. 3b).

A statistically significant difference at the 5% level was also observed between treatments. The



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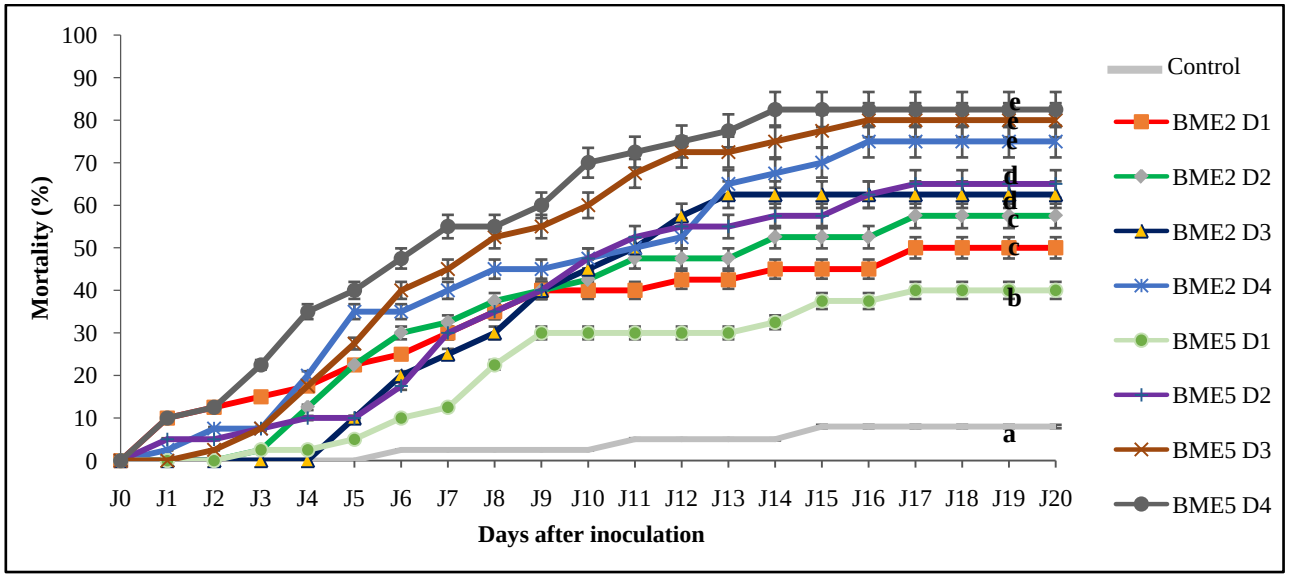


(a)

Fig. 3 Mortality rate according to the dose applied BME2 and BME5. (a) *P. sculptilis*; (b) *P. minor*.
With: D1 = 10^7 spores/mL; D2 = 10^8 spores/mL; D3 = 10^9 spores/mL; D4 = 10^{10} spores/mL;
(treatments marked with the same letter belong to the same homogeneous group).

Student-Newman-Keuls test yielded five distinct classes. With respectively 75%, 80% and 82.5% cumulative mortality, BME2 c = 10^{10} spores/mL, BME5 c = 10^9 spores/mL and BME5 c = 10^{10} spores/mL were the most virulent doses for *P. minor*. Then come BME5 c = 10^8 spores/mL and BME2 c = 10^9 spores/mL with respectively 65% and 75%. The low concentrations (c = 10^7 spores/mL and c = 10^8 spores/mL) of the BME2 isolate were less virulent compared to the previous ones (50% and 57.5%), but more effective than BME5 c = 10^7 spores/mL (40%) (Fig. 3b).

3.2.2 Rate of Dead Insects with Mycelial Down of *Metarhizium* Based on Applied Spore Concentrations

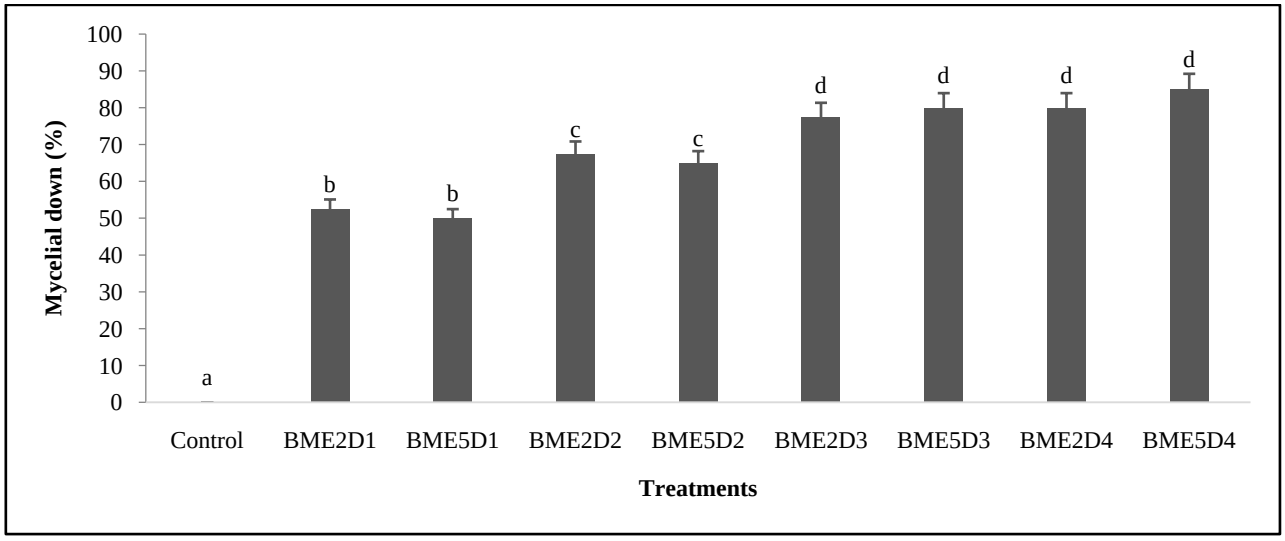
- *Prosoestus sculptilis*

In general, the percentage of *P. sculptilis* dead with mycelial down increases with the concentrations of *Metarhizium* spores applied. A significant difference was observed at the 5% threshold and four distinct

classes a, b, c and d were formed in increasing order of the rate of insects with a *Metarhizium* mycelial fuzz. Thus, the high concentrations (c = 10^9 spores/mL and c = 10^{10} spores/mL) of the BME2 and BME5 isolates with onset of mycelial down on 77.5% to 85% of the initial number of insects recorded the highest values. With 65% to 67.5% of the initial number of insects, the down of these two isolates appeared less with the concentration c = 10^8 spores/mL compared to the high concentrations, but more than with c = 10^7 spores/mL (50 to 52.5%). No myelial down of *Metarhizium* was observed in the control (Fig. 4a).

- *Prosoestus minor*

The percentage of *P. minor* deaths with mycelial down also increases with the concentrations of *Metarhizium* spores applied. A significant difference was observed between treatments and four distinct classes a, b, c, and d were formed in ascending order of the rate of insects with a *Metarhizium* mycelial fuzz. The high concentrations (c = 10^9 spores/mL and c = 10^{10} spores/mL) of the BME2 and BME5 isolates with 50% to 65% were those with whose down was observed



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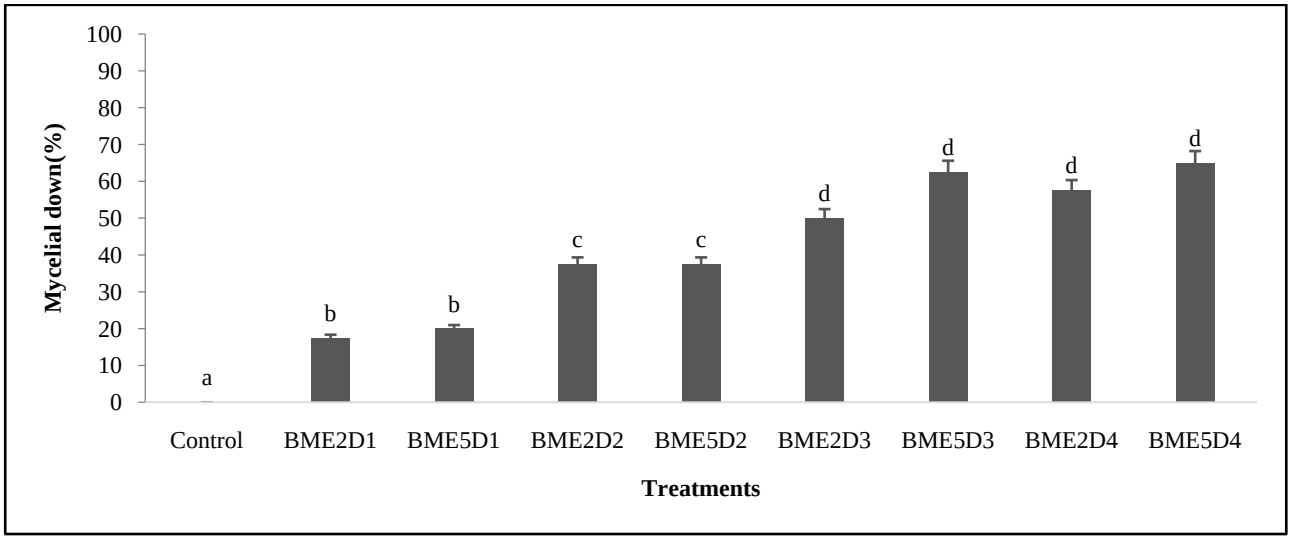

(b)

Fig. 4 Classification of BME2 and BME5 concentrations according to the rate of occurrence of mycelial fuzz. (a) *P. sculptilis*; (b) *P. minor*.

With: D1 = 10^7 spores/mL, D2 = 10^8 spores/mL, D3 = 10^9 spores/mL, D4 = 10^{10} spores/mL.

on the most insects. Next are the concentrations c = 10^8 spores/mL (37.5%) and c = 10^7 spores/ml (17.5 to 20%). No myelial down of *Metarhizium* was observed in the control (Fig. 4b).

3.2.3 Partial Conclusion

The BME2 and BME5 isolates were effective against *Prosoestus* spp. in the laboratory. The best results were obtained with concentrations C = 10^9 spores/mL and C = 10^{10} spores/mL without significant difference between these two concentrations. The field test was therefore performed with these two isolates at the concentration C = 10^9 spores/mL.

4. Discussion

The isolates of *Metarhizium anisopliae* screened in this study showed entomopathogenic potency against *Prososestus sculptilis* and *Prosoestus minor*, oil palm weevils, as they have been in the work of Ref. [11] on the black banana weevil *Cosmopolites sordidus* in Côte d'Ivoire. These three insect species belong to the same family (Curculionidae), which could explain this virulence of the fungus. These results are consistent with those of Ref. [12] who identified the genus *Metarhizium* as one of the main pathogens of the red palm weevil *Rhynchophorus ferrugineus*. Similarly, *Metarhizium anisopliae* var acridum IMI 330189, originally isolated from *Ornithacris cavroisi*, was tested on many locust species, revealing that it infects almost all locust species of the Acrididae and Pyrgomorphidae families, including *Schistocerca gregaria*, *Locusta migratoria*, *Locustana pardalina*, *Nomadacris septemfasciata*, *Oedaleus senegalensis* and other species of the Sahelian locust complex [13]. *Prosoestus sculptilis* was more susceptible to *Metarhizium* isolates compared to *Prosoestus minor*. This weevil, which is larger in size relative to *P. minor* [8] with elytrons bearing streaks materialized by large deep dots [14], would be more exposed to spores of the fungus by taking a larger quantity.

The BME2 and BME5 isolates showed a good level of efficacy in the inoculation of fungal weevils on fungal colonies. The 8- to 10-day LT50s are within the 4- to 90-day performance margin obtained in work in America and Africa reported by Ref. [15] on *Cosmopolites sordidus*, however LT90 and LT100 could not be reached in our study.

The results obtained with the spore suspension application of the two best isolates (BME2 and BME5) against *Prosoestus* spp. indicate that the maximum mortality rate of these insects is reached with the concentration of 10^9 spores/mL, which is therefore the optimum concentration. This concentration corresponds to that recommended by Refs. [16, 17] for the use of "Green Muscle", a bio-based pesticide based on a variety of *Metarhizium anisopliae*, in the control of the desert locust *Schistocerca gregaria*.

5. Conclusions

The fungus *Metarhizium anisopliae* has good virulence against *Prosoestus* spp. CNRA-BME2 and CNRA-BME5 were the most effective with an optimal dose of 10^9 spores/mL among the five isolates tested. This fungus was more virulent for *P. sculptilis* compared to *P. minor*. *Metarhizium anisopliae* is therefore a potential biological control agent to be considered in integrated control programs against *Prosoestus* species colonizing oil palm plantations.

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