Research Article ISSN 2641-4295

Food Science & Nutrition Research

Fungicidal Activity and Acute Toxicity of Three Elaborate Biopesticides Candidates for The Conservation of Fresh Kola Nuts

Jean-Marc N'GUESSAN[1*], James Halbin KOUADIO[1,2], Marc ADOU[1,3], N'Guessan Georges AMANI[1]

1Laboratory of Biochemistry and Technology of Tropical Products, UFR STA, Nangui Abrogoua University, Côte d'Ivoire.

2Research team of Plant Production Quality Management (GQPV), UFR Agroforestry. University Jean Lorougnon Guédé, Côte d'Ivoire.

3National Laboratory of Public Health. 52, Boulevard de Marseille, 18 BP 2403 ABIDJAN 18, Côte d'Ivoire.

***Correspondence:**
Jean-Marc N'GUESSAN, Laboratory of Biochemistry and Technology of Tropical Products, UFR STA, Nangui Abrogoua University, Côte d'Ivoire.

Received: 25 March 2019; **Accepted:** 22 April 2019

Citation: Jean-Marc N'GUESSAN, James Halbin KOUADIO, Marc ADOU. Fungicidal Activity and Acute Toxicity of Three Elaborate Biopesticides Candidates for The Conservation of Fresh Kola Nuts. Food Sci Nutr Res. 2019; 2(1): 1-9.

ABSTRACT

Description of the Subject: *The storage of fresh kola nut is a major concern in Côte d'Ivoire due to toxigenic molds proliferation.*

Objectives and Methods: *The aim of this study is to determine, in vitro, the antifungal effect of codified biopesticides "AG", "SI" and "TB" on Aspergillus flavus, Aspergillus niger and Penicillium sp., , Aflatoxinogenic and ochratoxinogenic molds, all by ensuring their high dose safety according to the OECD guideline 423 with Wistar rats.*

Results: *The results concerning the antifungal activity, showed that the mold strains are very sensitive to TB (inhibition rate; Ti > 75%) at the concentration of 7500 ppm, not very resistant or limited to SI (25% < Ti < 50%) and highly resistant to AG (Ti < 25%) even at high concentrations of 20000 ppm. Acute toxicity study results for biopesticides do not indicate any toxic effects at 2000 mg / kg of BW. However, at the dose of 5000 mg / kg of BW, only biopesticide TB was found biotolerated; AG and SI leading to toxic manifestations.*

Conclusion: *Taken as a whole, our results reveal that TB has a proven antifungal property and is biotolerized even at a high dose of 5000 mg / kg BW in the rat. It could therefore be recommended as a biopesticide for the conservation of kola nuts at the use rate of 7500 ppm.*

Keywords

Antifungal biopesticide, High dose safety, Kola nuts, Toxigenic molds.

Introduction

The kola tree (cola nitida) is a plant found in central and sub-Saharan Africa where it forms a large natural stand in the forest area [1]. Côte d'Ivoire is the world's leading producer and exporter, producing 260,747 tonnes of fresh nuts in 2016 [2]. Kola is mainly consumed fresh for these many uses, particularly as a stimulant, promoting physical and mental endurance and as ingredients of traditional African medicines [3,4]. It can also be used industrially in the formulation of certain pharmaceuticals and energy drinks. In fact, kola nut contains 2 to 3 % caffeine, 3.9 % tannin, 10.8 % protein, 1.6 % fat, 53 % carbohydrates and 3.5 % minerals [4].

However, conservation remains a real handicap for the kola industry in producing countries [5,6]. Losses vary between 10 and 80 % of production, mainly due to insects and mold during storage [7,8]. The mold species Aspergillus (*A. niger* and *A. flavus*) and Penicillium have been isolated from kola nuts after storage [9]. These molds producing mycotoxins, responsible for toxic manifestations proven in humans, represent real dangers for the consumer [10]. In order to control these pests of stocks, the actors of the sector often have recourse to various traditional conservation methods such as the use of leaves of Thaumatococcus danielli, and especially to the use of pesticides belonging to the organochlorine, organophosphorus families and pyrethroids [6].

The majority of organochlorine pesticides used are banned from sale and use in Côte d'Ivoire [11,12]. The presence of

organochlorine pesticides mainly belonging to the groups of hexachlorocyclohexanes (HCH), dichlorodiphenyltrichloroethane (DDT) and endosulfan at concentrations well above the maximum residue limits of organochlorine pesticides in foodstuffs recommended by the Codex Alimentarius is observed in 135 samples of kola nuts collected in the city of Anyama (Côte d'Ivoire) [13].

In addition, previous studies on the consumption of products treated with chemical pesticides have shown that they can lead to poisoning risks [14]. It is therefore necessary to look for other alternatives including the use of biopesticides that are not harmful to human health and the environment. Research on biopesticides for the conservation of kola nuts remains very limited yet several bioactive substances of plants have been found antifungal on mold species. Plant extracts of the family Lamiaceae, Euphorbiaceae, Zingiberaceae, Poaceae, Solanaceae and many others have shown their effectiveness on molds and some crop pests [15].

Plant extracts such as: *Ocimum gratissimum L.* (Lamiaceae), *Melaleuca quinquenervia* (Lamiaceae) have been shown to be effective on molds (*Fusarium oxysporum fsp* and *Pythium sp.*) [16,17]. In addition, antifungal properties of three local plants (Lippia multiflora, Boscia senegalensis and Ziziphus mucronata) against Puccinia arachidis, a fungus responsible for peanut rust have been shown [18]. Thus, several plant-based biopesticides are marketed in several African countries and are intended for different crops whose effectiveness in the conservation of kola nut should be evaluated.

Our study will therefore aim to evaluate the antifungal activity of three biopesticides developed and marketed in several African countries including Côte d'Ivoire. At the same time, the high dose safety of these three biopesticides has been studied to ensure consumer safety.

Materials and Methods

Material

Fungal Stumps: Three fungal strains (*Penicillium sp*., *Aspergillus flavus* and *Aspergillus niger*) isolated from kola nuts constituted the biological material. These strains were provided by the Mycology Laboratory of Pasteur Institute of Côte d'Ivoire.

Animal material: The animal material used was female rats (Rattus norvegicus, Muridae) of Wistar strain, nulliparous and non-pregnant. Animals from the Ecole Normale Supérieure (ENS) pet shop in Abidjan were between 7 and 9 weeks old and weighed between 127 and 143g of body weight (BW). Once collected, rats were acclimatized to the conditions of the animal house for one week before treatment. They were fed from the pellets of the company IVOGRAIN during the test. The relative temperatures and humidities of the pet shop were controlled. These values were 24 ± 1°C and 50 ± 5% Relative Humidity. In addition, a cycle of 12 hours of light and 12 hours of darkness was assured. The protocol for this study was approved by the Committee of Bioethics of Nangui Abrogoua University, Abidjan, Ivory Coast.

Culture medium and biopesticides used: Potato Dextrose Agar (PDA) culture medium was used for the cultivation of molds. The products used in this study concern 3 biopesticides. In particular AGRIMOR PESTOP 660 EC codified "AG", Silico sec codified "SI", and TopBio codified "TB" fungicide. These biopesticides are all sold on the local market.

Methods

Inhibition test for radial growth of molds: Five concentrations (5000, 7500, 10000, 15000 and 20000 ppm) of biofungicides were selected for this study, based on data from the data sheets of the different products and after several preliminary tests meeting the objective of defining the effective dose.

The biofungicides were incorporated into the PDA (Potato Dextrose Agar) culture medium after autoclaving at 121°C., 1 bar for 30 minutes, then poured at a rate of 20 ml into 9 cm diameter petri dishes. For each biofungicide concentration, five (5) Petri dishes were inoculated with a 7 mm diameter puck from the margin of a 4-day old mold culture and incubated at 25 ± 2 ° C at the photoperiod of 12 hours. Five molds control boxes were also produced without biofungicides. This experiment was repeated three (3) times.

Mycelial growth was assessed every 24 hours by measuring the average of 2 perpendicular diameters through the middle of the puck for six (6) days of incubation. The rate of inhibition (Ti) due to each biofungicide was evaluated on the sixth day of incubation with respect to mycelial growth in the control dishes according to the modified formula of Hmouni [19] :

$$Ti (\%) = [(Do - Dc) / Do] \times 100 \qquad (1)$$

Do: mycelial growth of the fungus (mm) on the control culture medium.
DC: mycelial growth of the fungus (mm) on the culture medium at the concentration (c) of the bio-fungicide.

According to Soro [17], the fungicide is said to : very active, when it has an inhibition rate (Ti) of between 75 and 100 %, the fungal strain is said to be very sensitive;
active, when it has a (Ti) between 50 and 75 %, the fungal strain is said to be sensitive;
moderately active, when it has a (Ti) between 25 and 50%, the strain is called limit;
little or not active, when it has a (Ti) between 0 and 25 %, the strain is said to be insensitive or resistant.

Study of acute oral toxicity

Distribution of rats in batch: The acute oral toxicity study was conducted according to the OECD guideline 423 for testing of chemicals [20]. Thus, three nulliparous and non-pregnant rats were used by biopesticide and stepwise. A control batch of three rats was also constituted in stages. Depending on the mortality and / or moribund status of animals, an additional step in the first was necessary to evaluate the acute toxicity of biopesticides. The initial

dose of 2000 mg / kg of BW was used in this study. Therefore, a total of 24 rats were used for the acute toxicity study of the three biopesticides.

Administration of doses of product to rats: The animals (rats) were kept at the pet shop of the Ecole Normale Supérieure (ENS) in Abidjan (Côte d'Ivoire) and placed in ventilated cages containing litter of wood chips renewed every three days at 08 AM. They were acclimatized to the conditions of the animal house for a week in their new cages before the treatment and fed from the pellets of the company IVOGRAIN. After subjecting the animals to fasting overnight (6:00 PM to 8:00 AM), the different biopesticides were administered orally at a dose of 2000 mg / kg body weight. The administration of the products was performed by gavage using a feeding tube and then each animal was weighed.

Observation of animals: After force-feeding with biopesticides, the animals are returned to their cages. After 2 hours, they had access to food again (pellets). They were observed immediately; then every 30 minutes for 8 hours, then once a day for 21 days. During this period, symptomatic disorders (agitation, lack of appetite, motor difficulties, drowsiness, sleep, convulsion, bent back, bristling hair etc ...) were noted, in the animals of the batch constituted. In accordance with paragraphs 9 and 21 of OECD Guideline 423, administration of next higher dose (5000 mg / kg of BW) to three additional animals was carried out to ensure the safety of the three biopesticides studied. At the end of the test, the animals that survived the different doses were weighed and then killed with humanity. The organs (liver, spleen, lungs, heart and kidneys) were carefully removed, then weighed for appreciation of their relative masses.

Monitoring of body mass and different organs of treated animals: The body mass of the animals was monitored before and after gavage by the biopesticide. The animals were weighed every 3 days at the same time (07: 00 AM). The mass is measured using a precision balance. The growth rate of rats after 15 and 21 days of treatment compared to the first day is expressed as a percentage and calculated according to the following formula:

$$\text{Growth rate (BW)} = [(Pj - Pj0) / Pj0] \times 100 \quad (2)$$

Pj_0: Weight of the first day; Pj: Weight of the day of interest.

Similarly, at the end of the experiment, the relative mass of the various organs (liver, kidneys, spleen, heart, lungs) is noted. This weight indicates the weight change of the organ with respect to the organism, following the administration of the pesticide. This mass is calculated according to the following formula:

$$\text{Relative mass} = (\text{Mass of organ} / \text{mass of animal}) \times 100 \quad (3)$$

Determination of serum parameters of acute toxicity: In order to confirm the results, the serum parameters of the treated rats were analysed. The blood sample was taken after 21 days of observation using a microfiber sampling. The rats having been previously anesthetized with chloroform. The blood of the different rats individually collected was used for the analysis of biochemical and hematological parameters.

Determination of hematological parameters: Hematological analysis was performed using an analyzer (URIT-2900 Plus). The parameters analyzed are: haemoglobin (HGB), red blood cells (RBC), white blood cells (WBC), haematocrit (HCT), blood platelet count (PLT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC), mean platelet volume (MPV), lymphocyte count (LYM), number of monocytes (MID), the number of granulocytes (GRA).

Determination of biochemical parameters: The biochemical parameters studied were determined using an analyzer (Robonik prietest touch). Assay methods for each biochemical parameter are described as directed by the reagent manufacturers. These methods use a centrifuge (UNIVERSAL 16 A) at 3000 round for 10 minutes to obtain serum.

Also, since the liver and kidneys are particularly sensitive organs to potentially toxic agents, their functions were examined at the end of the experiment. Renal function was assessed by serum creatinine and urea, whereas hepatic function was assessed by the enzymatic activities of serum glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and gamma-glutamyl transferase (gamma-GT). Blood glucose levels were also measured by enzymatic methods.

Statistical analyzes

After the different investigations, the information collected was processed using SPSS 22.0 statistical software. The data were subjected to an analysis of the variance (ANOVA) at a significance level of 5%. In case of significant difference, the means and standard deviations of the analyzed parameters were ranked against the control using Dunnett's t-test multiple-match test at the 5% threshold.

Results

Inhibition of the mycelial radial growth of *Penicillium sp*., *Aspergillus flavus* and *Aspergillus niger* by biopesticide AG, SI and TB

Inhibition rates varied with mold, biopesticides and biopesticide doses (Figures 1, 2 and 3). TB showed a significantly higher inhibition rate than AG and SI biofungicides. The efficacy threshold (50%) was largely achieved with the fungicide TB from the 7500 ppm concentration and on the 3 molds of the study. While this same threshold (50%) was reached with SI at 10,000 ppm and on *Penicillium sp* only. As for AG biofungicide, it showed no efficacy on these three molds.

The strains are said to be resistant to AG at the concentrations studied. In addition, at a dose of 5000 ppm, AG induced a stimulation of the mycelial growth of the 3 fungal strains studied.

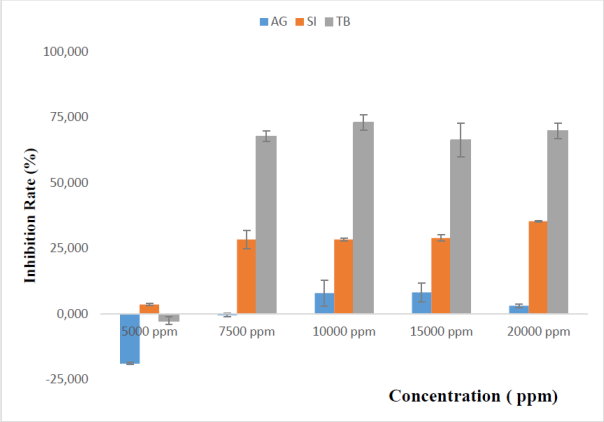

Figure 1: Evolution of the inhibition rate (Ti) of the mycelial radial growth of *A. flavus* at the sixth day of incubation, as a function of the doses of the 3 bio-fungicides.

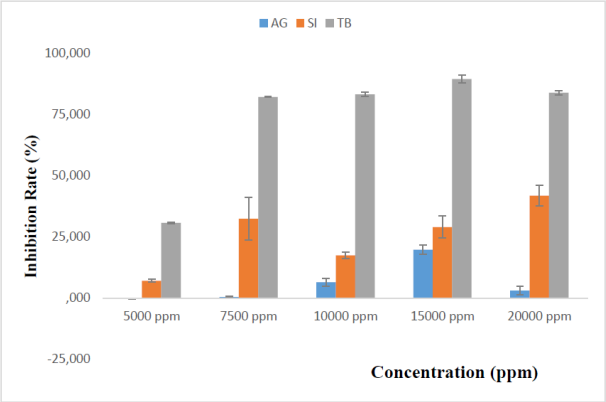

Figure 2: Evolution of the inhibition rate (Ti) of the mycelial radial growth of *A. niger* at the sixth day of incubation, as a function of the doses of the 3 bio-fungicides.

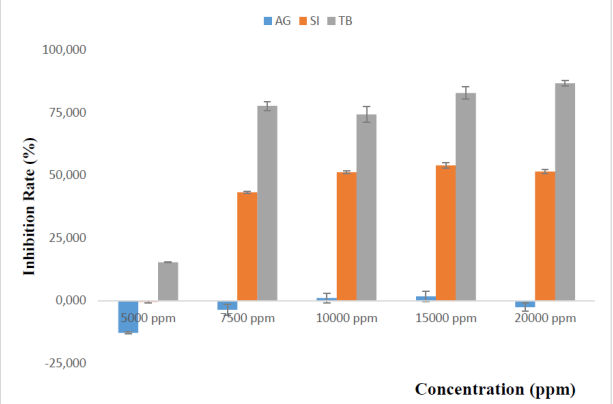

Figure 3: Evolution of the rate of inhibition (Ti) of the radial mycelial growth of *Penicillium sp* at the sixth day of incubation, as a function of the doses of the 3 bio-fungicides.

Toxicological studies of bio-pesticides

Response dose-mortality and clinical signs noted after gavage: Gavage administration of the biopesticides at a dose of 2000 mg / kg body weight to the different groups of established rats did not reveal any particular clinical signs during the 21 days of observation. While, the dose of 5000 mg / kg BW briefly resulted in some changes in the general appearance of the rats at certain batches during the 21 days of observation namely convulsion, lack of appetite and motor difficulties. In addition, at the dose of 5000 mg/kg of BW, the death of 3 rats, including 1 rat in the group of rats fed by AG and 2 rats in the group of fed rats SI was observed during 21 days of observation.

While no mortality was observed in the lot treated with TB. Table 1 shows rat mortality by biopesticide at 5000 mg / kg CP. At the level of the group fed by AG, the death of a rat was observed on the ninth day after force-feeding. As for the group fed by SI, the death of 2 rats, one on the first day and the other on the fifth day was observed during the observation. These deaths were preceded by some clinical signs previously indicated.

According to the Globally Harmonized System (GHS), the biopesticides AG and SI are in Category 5. Their lethal dose 50% (LD50) is greater than 2000 mg / kg body weight. While the biopesticide TB is in the unclassified category, its lethal dose 50% (LD50) is greater than 5000 mg / kg body weight (Table 1) .This value of the LD50 obtained over 21 days of observation , indicates that the products AG, SI and TB, administered orally, are non-toxic, in animals treated under the conditions of this study.

Dose (mg/kg of BW)	Number of deaths per batch			
	Control	AG	SI	TB
2000	0	0	0	0
5000	0	1	2	0

Table 1: Mortality of rats by dose of bio-pesticides.

Body mass of treated animals: Gavage of the female rats by the biopesticides AG, SI and TB at a dose of 2000 mg / kg BW, did not cause any major changes in body weight compared with controls (Figure 4). Indeed, the statistical analysis of ANOVA variance of the growth rates on day 15 and day 21 showed that there is no difference between the lots fed by the bio-pesticides and the control group at the threshold of 5 %, (Tables 2).

Traitement	Control	Agrimor	Silico sec	TopBio	ANOVA Table	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	F	p-value
Growth rate J15	24.71 ± 8.17	23.35 ± 1.36	20.04 ± 7.07	21.16 ± 2.80	0.422	0.743
Growth rate J21	28.77 ± 6.77	23.53 ± 1.93	22.65 ± 4.45	20.99 ± 5.16	1.414	0.308

Table 2: Growth Rates after 15 and 21 Days of Treatment at the 2000 mg / kg dose of BW. Mean ± standard deviation, n = 3.

In addition, the dose of 5000 mg / kg of PC resulted in a decrease in body weight which even led to the death of a rat in the batch treated with Agrimor (AG) and two rats in the lot treated with Silico sec (SI). While no major changes in body weight compared to controls were observed in the batch treated with TopBio (TB) at the same dose of 5000 mg / kg of PC (Figure 5). Indeed, analysis of variance (ANOVA) showed that there is no difference between the batch gaved by TB and the control group at the 5% threshold,

(P = 0.052 and 0.597) respectively for the 15th and 21st day of experimentation (Table 3).

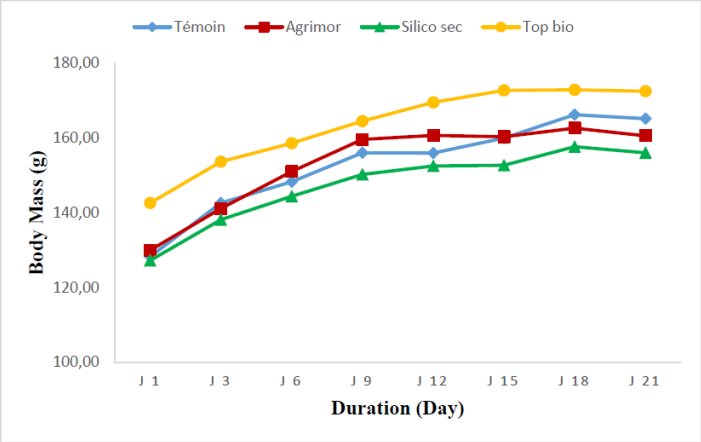

Figure 4: Evolution of body mass during 21 days in animals fed by bio-pesticides at a dose of 2000 mg / kg BW.

Traitement	Control	TopBio	ANOVA Table	
	Mean ± SD	Mean ± SD	F	p-value
Growth rate J15	26.39 ± 2.54	22.04 ± 1.02	7.532	0.052
Growth rate J21	30.10 ± 3.34	28.52 ± 3.42	0.329	0.597

Table 3: Growth Rates after 15 and 21 Days of Treatment at a Dose of 5000 mg / kg BW. Mean ± standard deviation, n = 3.

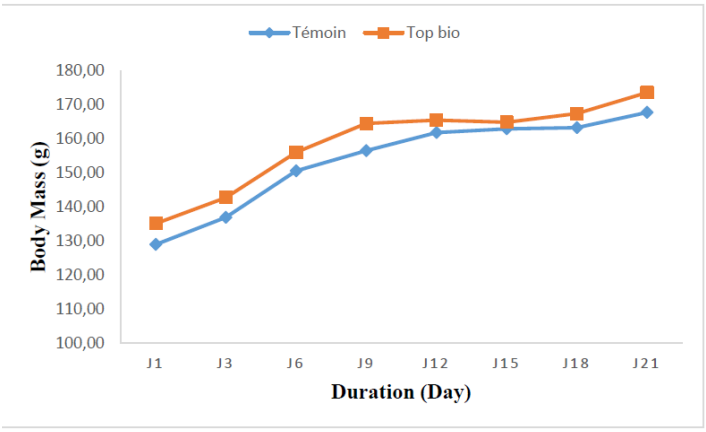

Figure 5: Evolution of the body mass during 21 days in the animals treated with TB at the dose 5000 mg / kg of BW.

Masses of different organs: After macroscopic examination of rat organs, no morphological changes were observed in the organs of the rats fed with the 3 bio-pesticides at a dose of 2000 mg / kg CP in comparison with those of the control rats, as well as the batch gaved by TB at 5000 mg / kg of PC.

The average of the relative mass of the different organs of the female rats fed with the 2000 mg / kg dose of the 3 bio-pesticides shows no significant increase in the relative weight of the kidneys, lungs, liver, spleen and of the heart after 21 days of observation, as well as the batch treated with TB at 5000 mg / kg of BW (Tables 4 and 5).

Variables	Control	Agrimor	Silico sec	TopBio	ANOVA Table	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	F	p-value
Liver (gr)	5.43 ± 0.19	5.09 ± 0.37	4.82 ± 0.64	5.89 ± 0.57	2.878	0.103
Heart (gr)	0.63 ± 0.08	0.61 ± 0.08	0.55 ± 0.05	0.71 ± 0.13	1.684	0.247
Lung (gr)	0.96 ± 0.07	1.41 ± 0.57	1.05 ± 0.12	1.37 ± 0.33	1.315	0.335
Spleen (gr)	0.37 ± 0.03	0.34 ± 0.02	0.31 ± 0.04	0.52 ± 0.18	2.884	0.103
Kidneys (gr)	0.81 ± 0.07	0.80 ± 0.05	0.78 ± 0.02	0.94 ± 0.06	2.283	0.156

Table 4: Relative masses (g) of the various organs after 21 days of treatment at a dose of 2000 mg / kg of BW. Mean ± standard deviation, n = 3.

Variables	Control	TopBio	ANOVA Table	
	Mean ± SD	Mean ± SD	F	p-value
Liver (gr)	5.63 ± 0.75	5.73 ± 0.1	0.056	0.825
Heart (gr)	0.59 ± 0.03	0.67 ± 0.04	6.393	0.065
Lung (gr)	1.12 ± 0.12	1.15 ± 0.13	0.087	0.783
Spleen (gr)	0.41 ± 0.1	0.44 ± 0.07	0.180	0.693
Kidneys (gr)	0.82 ± 0.07	0.93 ± 0.05	4.826	0.093

Table 5: ANOVA relative masses (g) of the different organs after 21 days of treatment at the dose of 5000 mg / kg of BW. Mean ± standard deviation, n = 3.

Variation of biochemical parameters: ANOVA statistical analyzes performed at the 5% threshold, after determination of glycemia, glutamic oxaloacetic transaminase, glutamic-pyruvic transaminase and gamma glutamyl transferase, show that bio-pesticides have no effect significantly on lots treated at 2000 mg / kg of BW compared to the control (Table 6). Whereas for these same biochemical parameters, the ANOVA test at the 5% threshold showed a significant difference only after the measurement of gamma glutamyl transferase in the batch gaved with TB at 5000 mg / kg of BW compared with the control (Table 7).

Parameters	Control	Agrimor	Silico Sec	TopBio	ANOVA Table	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	F	p-value
Urea (g/l)	0.51 ± 0.01	0.52 ± 0.08	0.53 ± 0.04	0.47 ± 0.01	0.943	0.464
Glycemic Index (g/l)	0.97 ± 0.13	1.15 ± 0.17	0.96 ± 0.06	1.06 ± 0.11	1.441	0.301
Creatinine (mg/l)	6.02 ± 0.34	7.07 ± 1.5	5.66 ± 0.46	7.1 ± 1.36	1.471	0.294
GOT (UI/L)	271.33 ± 86.13	227.53 ± 0.59	238.1 ± 40.1	197.07 ± 35.98	1.091	0.407
GPT (UI/L)	156.6 ± 41.12	109.43 ± 4.76	144.57 ± 5.63	101.63 ± 25.86	3.534	0.068
Gamma-GT	16.93 ± 4	18.97 ± 7.93	22.75 ± 3.23	21.15 ± 2.79	0.799	0.528

Table 6: Biochemical parameters after 21 days of treatment with the biopesticide at the dose of 2000 mg / kg of BW compared to the control.

Mean ± standard deviation, n = 3.
GPT: glutamic pyruvic transaminase; GOT: glutamic oxaloacetic transaminase.

Parameters	Témoin	TopBio	ANOVA Table	
	Mean ± SD	Mean ± SD	F	p-value
Urea (g/l)	0.48 ± 0.02	0.48 ± 0.06	0.006	0.942
Glycemic Index (g/l)	1.09 ± 0.27	0.90 ± 0.19	0.979	0.379
Creatinine (mg/l)	5.36 ± 0.41	5.65 ± 0.45	0.679	0.456
GOT (UI/L)	263.67 ± 68.39	243.93 ± 47.12	0.169	0.702
GPT (UI/L)	150.40 ± 13.32	133.70 ± 3.25	4.45	0.103
Gamma-GT	18.91 ± 4.82	38.34 ± 6.48	17.34	0.014

Table 7: ANOVA biochemical parameters after 21 days of treatment with TB at the dose of 5000 mg / kg of BW compared to the control. Mean ± standard deviation, n = 3.
GPT: glutamic pyruvic transaminase; GOT: glutamic oxaloacetic transaminase.

Hematological parameters

The hematological results of the ANOVA statistical analyzes obtained are shown in Table 8. They reveal that there is no significant change in the haematological parameters of the animals fed by the 3 bio-pesticides at a dose of 2000 mg / kg of BW compared to the control. However, a difference at the 5% threshold is observed at the Hematocrit (HCT) parameter, (F = 4.95 ; P = 0.03). In addition, statistical analysis of multiple Dunnett t-test comparisons at the 5% threshold for the 2000 mg / kg dose of BW reveals that there is no significant difference between Hematocrites in treated rats compared to control (Table 9). On the other hand, goading by TopBio at a dose of 5000 mg / kg of BW for these same haematological parameters, the ANOVA statistical analysis shows a significant difference at the threshold of only 5% for the Granulocyte (GRA) parameter, (F = 10.18 ; P = 0.033), (Table 10).

Variables	Control	Agrimor	Silico Sec	Top bio	ANOVA Table	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	F	p-value
WBC (x 10^3/µL)	17.27 ± 1.19	13.73 ± 3.75	14.98 ± 3.35	18.13 ± 5.52	0.86	0.50
LYM (x 10^3/µL)	11.52 ± 1.06	8.42 ± 1.60	10.23 ± 2.65	11.34 ± 4.78	0.73	0.57
MID (x 10^3/µL)	1.42 ± 0.51	1.04 ± 0.33	1.21 ± 0.12	1.76 ± 0.65	1.43	0.31
GRA (x 10^3/µL)	4.33 ± 0.65	4.32 ± 1,89	3.54 ± 0.72	5.02 ± 2.85	0.35	0.79
LYM (%)	66.70 ± 3.61	61.77 ± 5.55	68.00 ± 2.91	62.43 ± 13.57	0.49	0.7
MID (%)	8.07 ± 2.35	7.57 ± 0.31	8.27 ± 1.19	9.53 ± 0.81	1.10	0.41
GRA (%)	25.23 ± 4.51	30.67 ± 5.35	23.73 ± 2.78	28.03 ± 13.38	0.48	0.71
RBC (x 10^6/µL)	6.18 ± 0.51	6.10 ± 0.25	6.78 ± 0.24	5.87 ± 0.31	3.87	0.06
HGB (g/dL)	12.70 ± 0.85	12.03 ± 0.25	13.47 ± 0.55	11.63 ± 1.00	3.69	0.06
MCHC (g/dL)	30.33 ± 0.42	29.63 ± 0.59	29.63 ± 0.70	29.03 ± 1.76	0.82	0.52
MCH (pg)	20.53 ± 0.99	19.73 ± 0.42	19.87 ± 0.12	19.73 ± 0.81	0.97	0.46
MCV (fL)	67.83 ± 2.97	66.60 ± 0.20	66.97 ± 1.17	68.20 ± 3.25	0.32	0.81
HCT (%)	41.83 ± 2.21	40.57 ± 1.53	45.43 ± 1.77	40.03 ± 2.00	4.95	0.03
PLT (x 10^3/µl)	843.00 ± 175.86	931.00 ± 278.91	981.00 ± 22.61	937.67 ± 161.30	0.3	0.83
MPV (fL)	8.43 ± 0.40	8.27 ± 0.50	9.77 ± 1.54	9.37 ± 0.50	2.08	0.18

Table 8: ANOVA of NFS at 2000 mg / kg of BW.
Mean ± standard deviation, n = 3.
HGB: Haemoglobin, RBC: Red Blood Cells, WBC: White Blood Cells, HCT: Haematocrit, PLT: Blood Platelet, MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Haemoglobin, MCHC: Mean Corpuscular Haemoglobin Concentration, MPV: Mean Platelet Volume, LYM: Lymphocytes, MID: Number of Monocytes, GRA: Granulocytes.

Variable	Traitement	Traitement Mean ± SD	Control Mean ± SD	p-value
HCT (%)	Agrimor	40.57 ± 1.53	41.13 ± 2.21	0.76
	Silico Sec	45.43 ± 1.77		0.11
	Top bio	40.03 ± 2.00		0.54

Table 9: Multiple Comparisons of Dunnett's T-Test for the 2000 mg / kg dose of BW.
Mean ± standard deviation, n = 3 ; HCT: haematocrit.

Variables	Control	TopBio	ANOVA Table	
	Mean ± SD	Mean ± SD	F	p-value
WBC (x 10^3/µL)	15.88 ± 4.36	16.42 ± 2.55	0.034	0.862
LYM (x 10^3/µL)	10.50 ± 3.77	10.10 ± 2.01	0.027	0.878
MID (x 10^3/µL)	1.45 ± 0.58	1.70 ± 0.26	0.47	0.531
GRA (x 10^3/µL)	3.93 ± 0.22	4.62 ± 0.30	10.18	0.033
LYM (%)	64.90 ± 7.61	61.20 ± 2.96	0.616	0.476
MID (%)	8.97 ± 1.42	10.40 ± 0.52	2.688	0.176
GRA (%)	26.13 ± 8.12	28.40 ± 2.79	0.209	0.671
RBC (x 10^6/µL)	6.54 ± 0.48	6.22 ± 0.28	1.024	0.369
HGB (g/dL)	12.90 ± 0.36	12.50 ± 0.53	1.171	0.34
MCHC (g/dL)	27.93 ± 1.00	28.47 ± 1.20	0.349	0.587
MCH (pg)	19.73 ± 1.14	20.17 ± 1.59	0.147	0.721
MCV (fL)	70.70 ± 1.91	70.90 ± 3.03	0.009	0.928
HCT (%)	46.17 ± 2.22	44.03 ± 1.40	1.977	0.232
PLT (x 10^3/µl)	1078.00 ± 179.89	804.67 ± 169.19	3.675	0.128
MPV (fL)	8.53 ± 1.55	7.97 ± 0.47	0.367	0.577

Table 10: ANOVA of NFS at 5000 mg / kg of BW.
Mean ± standard deviation, n = 3.
HGB: Haemoglobin, RBC: Red Blood Cells, WBC: White Blood Cells, HCT: Haematocrit, PLT: Blood Platelet, MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Haemoglobin, MCHC: Mean Corpuscular Haemoglobin Concentration, MPV: Mean Platelet Volume, LYM: Lymphocytes, MID: Number of Monocytes, GRA: Granulocytes.

Discussion

Radial mycelial growth of *Penicillium sp*., *Aspergillus flavus* and *Aspergillus niger* was significantly inhibited by TB from 7500 ppm. This inhibition was much greater than that of the biopesticides SI and AG. TB exhibited more effective in vitro fungicidal activity than did SI and AG. According to the classification scale of Soro [17], TB is very active on *Aspergillus niger* and *Penicillium sp.*, and active on *Aspergillus flavus* from 7500 ppm. In addition, the efficacy of TB at different doses remained very marked with a statistically uniform inhibitory effect on the three fungal species. This could be explained by the sensitivity of mold *Aspergillus flavus*, *Penicillium sp.* and *Aspergillus niger* with respect to biopesticide TB [17].

These observations confirm the antifungal properties of TB in addition to its insecticidal activity. Although TB did not completely inhibit mycelial growth of three molds mentioned above, it had a variable toxic effect depending on the concentrations and mold strains. The effectiveness of TB may depend on the chemical compounds it contains. Indeed TB contains azadirachtin, nimbin, citronellal, citronellol and geraniol as bioactive compounds.

These results confirm those presented by Triki [21] who showed that phenolic compounds (geraniol), have a strong antifungal activity against *Aspergillus niger*. Also, the plurality of secondary metabolites could increase the rates of inhibition of mycelial growth by their synergistic effects [16,22,23]. According to Kassi [24], the phenolic compounds are recognized as toxic and would target the envelopes of microorganisms such as the cytoplasmic membrane and the wall. Their action is also associated with the inhibition of germination of propagation organs (spores). The work of Hamilton and Archbold [25] have also shown that aldehydes such as citronellal alter the permeability of membranes. Azadirachtin molecule would also be important in reducing mycelial growth by its action as an anorexic agent [26].

Results obtained by this study of inhibition of mycelial growth with use TB are more convincing to those of fungicides AG and SI. This high sensitivity of the strains of *Aspergillus flavus*, *Aspergillus niger* and *Penicillium sp.*, To biopesticide TB may be of interest for the treatment of kola nuts at 7500 ppm.

The evaluation of the acute toxicity of the biopesticides AG, SI and TB on rats at 2000 mg / kg of BW, showed that these biopesticides are not toxic to the tested organs. However, it should be noted that they led to the appearance of some transient clinical signs in the batches. In addition, evaluation of the acute toxicity of these three biopesticides on rats at 5000 mg / kg of BW, showed that TB is less toxic than AG and SI. In fact, mortalities have been observed in these last batches.

All these disorders and mortalities observed are due to the defense response of the living organism against foreign molecules (secondary metabolites) received after administration of biopesticides AG, SI and TB at high doses. The animals that survived and found normal appearance are those that would probably have had ability to metabolize the bioactive compounds of biopesticides. Moreover, the body masses of treated animals evolve differently over time compared to the control animals. According to the statistical analysis of the data, there are no significant effects in the administration of AG, SI and TB biopesticides on the mass of the different organs (liver, kidney, spleen, heart and lung) of the animals treaties.

Biopesticides administered at the dose of 2000 mg / kg of BW, caused no mortality in the constituted batches, as well as TB at the dose of 5000 mg / kg of PC. These high doses of TB are therefore biotolerated. Thus, according to the globally harmonized classification system (GHS) (OECD 423, 2001), the LD 50 of TB is greater than 5000 mg / kg of BW after 21 days of observation in rats under the conditions of this study. While the LD 50 of AG and SI is greater than 2000 mg / kg of BW after 21 days of observation in rats under the same study conditions.

The biochemical and hematological parameters analysed to demonstrate the non-toxicity of the biopesticides, show that the average values of transaminases GPT and GOT, gamma-GT and Glycemic Index remained within normal limits in experimental animals compared to controls. This reflects normal carbohydrate metabolism and further that no abnormality was induced in the liver during this test [27]. Creatinine and urea are excellent markers of renal function, their increase or decrease reflects renal dysfunction [28]. These levels do not vary in rats receiving the 2000 mg / kg dose of body weight biopesticides and the dose of 5000 mg / kg of BW Top bio, indicating normal kidney function.

Hematological examination indicates different results. The results of the statistical analyzes show that administration of high dose (2000 mg / kg of BW) of AG, SI and TB biological fungicides does not cause a significant modification at 5% threshold. The same analyzes show that ingestion of TB at the dose of 5000 mg / kg of BW does not cause significant variations in the levels of the hematological parameters evaluated. Many physiological mechanisms reflected by these hematological parameters (respiration, immunology, etc.) therefore remain normal in these treated rats with AG, SI and TB biopesticides [28].

Conclusion

The antifungal power of AG, SI and TB biopesticides as well as their safety were evaluated for the good post-harvest conservation of kola nut. The biopesticide TB is very active on the fungal strains of kola nut and not toxic. First, its application showed obvious antifungal activity with superior performance compared to other fungicides. In fact, the inhibition rates in *A. flavus* reached respectively 67.78%, 72.89% and 66.24% and 69.82% respectively at the doses: 7500 ppm, 10000 ppm, 15000 ppm and 20000 ppm. The inhibition rates in *A. niger* reached 82.19 %, 83.26 %, 89.49 % and 83.91 % respectively for these same doses above. *Penicillium sp.* inhibition levels were 77.67%, 74.27%, 82.77% and 86.65% respectively for the doses: 7500 ppm, 10000 ppm, 15000 ppm and 20000 ppm.

The dose of 7500 ppm would be the most economical because this dose of TB is active on *A. flavus*, very active on *A. niger* and *Penicillium sp*.,. In addition, the biopesticide TB is less toxic than AG and SI. The dose 5000 mg / kg of BW did not cause mortality in the experimental rats and had no significant effect on the relative mass of the organs. Biochemical markers of vital organs and hematological parameters confirm the safety of TB observed in preclinical manifestations. Its safety is therefore proven in view of the tests.

The choice of biopesticide TopBio (TB) is therefore advantageous for the conservation of the kola nut. It could be recommended at the use rate of 7500 ppm.

Acknowledgment

The authors would like to thank the Interprofessional Fund for Agricultural Research and Council (FIRCA, Projet N° 063/ FIRCA/LBATPT/FILIERE COLA/2016) in Côte d'Ivoire, which financed this study. They would also like to thank Dr. GBOGBO Moussa and M. KOFFI Yao Stéphane for their technical support.

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