**Journal of Plant Sciences**
2023; 11(6): 171-181
http://www.sciencepublishinggroup.com/j/jps
doi: 10.11648/j.jps.20231106.11
ISSN: 2331-0723 (Print); ISSN: 2331-0731 (Online)

# Biofungicide Based Calcium, *Azadirachta indica* and *Sida acuta* Against *Phytophthora megakarya*

**Ebenezer Tatiekam Foka[1, 2], Paul Martial Téné Tayo[1, *], Tatiana Flore Magni Pacha[1, 2], Aristide Dzelamonyuy[1, 3], Sylvain Leroy Kamdem Sado[4], Pierre Effa Onomo[1, 2], Cécile Annie Ewané[1, 2, *], Thaddée Boudjeko[1, 2]**

[1]Laboratory of Phytoprotection and Valorization of Genetic Resources, Biotechnology Centre-Nkolbisson, Yaounde, Cameroon

[2]Department of Biochemistry, Faculty of Science, University of Yaoundé 1, Yaoundé, Cameroon

[3]Biochemistry and Molecular Biology, Indiana University School of Medicine, Michigan, USA

[4]Department of Microbiology, Faculty of Science, University of Yaounde I, Yaounde, Cameroon

## Email address:

martialtene@gmail.com (Paul Martial Téné Tayo), ccilany.ewane@gmail.com (Cécile Annie Ewané)

[*]Corresponding author

## To cite this article:

Ebenezer Tatiekam Foka, Paul Martial Téné Tayo, Tatiana Flore Magni Pacha, Aristide Dzelamonyuy, Sylvain Leroy Kamdem Sado, Pierre Effa Onomo, Cécile Annie Ewané, Thaddée Boudjeko. Biofungicide Based Calcium, *Azadirachta indica* and *Sida acuta* Against *Phytophthora megakarya*. *Journal of Plant Sciences*. Vol. 11, No. 6, 2023, pp. 171-181. doi: 10.11648/j.jps.20231106.11

**Received**: September 14, 2023; **Accepted**: October 4, 2023; **Published**: November 9, 2023

**Abstract:** Plant pesticides are a complementary means to other control methods and an alternative to chemical control. The identification of potential plants that can be used in plant protection against pests is increasing enormously, but the availability of bioformulations is low. The new products developed for plant protection aim to protect them against a larger range of pathogens and include elicitors. The objective of this study is to produce an optimal emulsion bioformulation based on neem (*Azadirachta indica* A. Juss.), Sida (*Sida acuta* Burm. F.) and calcium, that can be used for plant protection against pests. The surface plot of the four factor simplex lattice designs data help to construct significant linear models for stability and activity. The best formulation was selected and optimize. The four Factor mixture designs and stability as well as activity models showed that the best formulation has neem oil (No) 12% (v/v), neem aqueous extract (Ne) 9% (w/v), sida weed hydroalcoholic extract (Se) 9% (w/v), Oyster shell's calcium-rich extract (Ca) 6% (w/v) and tween-80 (Tw) 24% (v/v) with the production process "AQ + (Tw+No)". Therefore, the optimize formulation has No 12% (v/v), Ne 9% (w/v), Se 9% (w/v), Ca 4.5%(w/v), calcium oxide (CaO 1.5% (w/v) and Tw 24% (v/v) with the production process "(Tw+No) + AQ". The best formulation and the optimize one at ambient temperature have 100% of stability and a significant dose dependent activity ($P$ <0.05) against plant pathogens. Apply optimize formulation at 1% on the detached cocoa leaves before inoculation with *Phytophthora megakarya* reduces disease severity index from 4.2 to 1.5. These results suggest that our models and bioformulation can be useful for *T. cocoa* protection against *P. megakarya*, the causal agent of black pod disease.

**Keywords:** *Theobroma cocoa* L., *Phytophthora megakarya*, Bioformulation of Fungicide, Neem, Sida Weed, Oyster Shell

## 1. Introduction

Cocoa (*Theobroma cacao* L.) is one of the main cash crops in tropical countries. Cocoa production in Cameroon contributes to the national Gross Domestic Product (GDP) by 1.2% and generates annual revenue of over €400 million, providing more than 400,000 jobs [1]. However, its cultivation is facing many problems among which parasitic attacks such as black pod disease caused by the soil borne fungal pathogen *Phytophthora megakarya* [2]. The use of chemical fungicides is often necessary to counter this problem despite the fact that it creates many problems, including the emergence of resistant pathogens, soil biodegradation and human health. It is therefore necessary to explore environmentally safe and effective alternative methods of control. In recent decades, prospects for biological control of black pod disease have been regularly explored [3-5]. Improving the formulation of natural bioactive agents like

microorganisms and plants extracts was therefore an important next step towards optimization of this biological control strategy. A new dynamic is trying to combine biocontrol agents and "inert" materials in formulations for pest management [6].

Among the plants used by indigenous peoples and now included in formulation worldwide, neem and sida weed have a great place. Indeed, on the one hand, neem (*Azadirachta indica* A. Juss.) is a valuable source of compounds, such as azadirachtin, which can be used for the development of drugs or biopesticides [7]. Neem oil amplifies pesticidal properties with its composition which contain sulfurous compound [7]. Many neem-based formulations exist but, the problem with these formulations is their instability under natural conditions [8]. In the other hand, *Sida acuta* Burm. F. (Malvaceae) is a small, erect, branched perennial herb or small shrub about 1.5 m tall [9]. It grows abundantly on cultivated fields, wastelands and roadsides in Cameroon, where it is called "sengh" in the West of the country and its common name is sida [10]. Many authors have demonstrated the antimicrobial activity of ethanolic, hot and cold-water extracts of *S. acuta* [11, 12]. Additionally, [13] study the antimicrobial potential of *Sida acuta* leaf extract nanoparticles.

The oyster shell is the waste product of the oyster and consists mainly of calcium carbonate and chitin [14]. In the previous work, we demonstrated that application of oyster shell powder stimulated the resistance of *T. cacao* in its interaction with *P. megakarya* [4]. This stimulation could be correlated to the component of oyster shell powder such as calcium. Calcium is a key nutrient for plant with significant functions including structural roles in the plant cell wall providing mechanical strength for normal transport and retention of other elements [15, 16]. When plants are exposed to a stressful situation, a rapid increase in cytosolic $Ca^{2+}$ occurs, which is a key factor in the expression of stress-responsive genes and physiological responses of plant cells to stressful conditions as extreme temperatures, drought, salinity and pathogenic attack. Changes in cytosolic $Ca^{2+}$ concentrations are often closely related to the severity of the stress [17].

This investigation was carried out to produce and optimize a bioformulation based on neem, *Sida acuta* and calcium-rich extracts of oyster shells, usable against *Phytophthora megakarya*. The stability of bioformulation was studied and the selected formulation was used in a simplex lattice design. Then, the stability and activity model were built from the data of the simplex-lattice experiment and the best formulation was selected and optimized by correcting its pH.

# 2. Materials and Methods

### *2.1. Materials*

Neem kernels were obtained after shelling the dry neem seed, collected from urban and peri-urban zones of Maroua (Far North Region, Cameroon). Neem oil (No) was extracted by manual pressure on powder obtain after grinding the dry kernels in electrical blender. Neem aqueous extract (Ne) was obtained according to the protocol of F. Lesueur [18].

The sida plants were collected from urban and peri-urban areas of Yaoundé (Centre Region, Cameroon). Extraction was done twice and its extract was obtained according to J. R. Kuiate [19]. A hydroalcoholic solution (1 volume of water and 0.5 volume of ethanol 95%) and air-dried plants powder were used for extraction. 100 g of powder was put into a conical flask containing 600 ml of sterile distilled water and left to macerate for 24 h at room temperature. The resulting mixture was then filtered using a piece of cloth and Whatman N° 2 filter paper, then the filtrate was lyophilized and stored at room temperature.

The oyster shells from Mouanko (Littoral Region, Cameroon) were carefully washed in tap water and air-dried. Calcium-rich oyster shells extract was obtained according to [20] with modifications. Briefly, 2 kg of oyster shell were heated at 200°C for one hour to facilitate the grinding. They were then ground in a grinding machine (MS 20B grinding machine), and then sieved using a 0.8 mm sieve to obtain the finest powder that was heated in an oven at 1000°C for 1h. Ash obtains was dissolved in 25% HCl (v/v) in the proportion 1/15 (w/v). Soluble compounds were collected by filtration and the solvent evaporated in an oven at 115°C. The calcium-rich oyster shells extract (Ca) was store at room temperature.

*Fusarium oxysporum*, *Pythium myriotylum*, *Phytophthora infestans, Phytophthora megakarya* isolates used in this study were obtained from the microorganism bank of the Laboratory of Phytoprotection and Valorisation of Genetic Resources (LPVGR) of the Biotechnology Centre of the University of Yaoundé 1, Cameroon.

### *2.2. Process of Bioformulation of the Emulsion Product*

The bioformulation process was done in two steps and followed by an optimization by correcting its pH.

In the first step, emulsification process of No was carried out using a quasi-experimental design which can be subdivided in three groups (Table 1). This design has in the first group three oil concentration 6%; 9% and 12% (v/v); and nine surfactant Tween 80 (Tw) concentration 0%; 1%; 3%; 6%; 9%; 12%; 18%; 21% and 24% (v/v) that were used with fixed amount of Ne 9% (w/v), Se 9% (w/v) and Ca 6% (w/v). Each aqueous soluble extract was dissolved in water then Tw and No were added and the whole was mixed using a vortex at 1000 rpm for one minute to form emulsion. The stability of the emulsions was observed for 48 h.

In the second group, the emulsion was made by changing the surfactant concentration while Se was absent. Fixed among No (12% v/v), Ne (18% w/v), and Ca (6% w/v) were used while Tw were used at 0%, 12% and 21% (v/v). In the third group of this quasi-experiment design, the emulsion was made using the surfactant concentration of 0%, 12% and 21% (v/v) while Ne was absent (0% w/v). The fixed among of No 12% (v/v), Ne 0% (w/v), Se 18% (w/v) and Ca 6% (w/v) were used. The emulsion (2 mL) was prepared and stored at 26 °C in a 5 mL test tube and the stability was determined by

observing the phase separation in 48 h. The stability scale was as follow: (1) 0% for oil flocculation, 25% for milky medium with oil flocculation; (2) 50% for milky medium with cream and oil flocculation, (3) 75% for milky medium with cream flocculation, and (4) 100% for milky medium without oil or cream separation.

***Table 1.*** *Quasi-experiment design data.*

| Essai codes | No v/v [%] | Tw v/v [%] | Ne w/v [%] | Se w/v [%] | Ca w/v [%] |
|---|---|---|---|---|---|
| 1A | 6 | 0 | 9 | 9 | 6 |
| 2A | 6 | 1 | 9 | 9 | 6 |
| 3A | 6 | 3 | 9 | 9 | 6 |
| 4A | 6 | 6 | 9 | 9 | 6 |
| 5A | 6 | 9 | 9 | 9 | 6 |
| 6A | 6 | 12 | 9 | 9 | 6 |
| 7A | 6 | 18 | 9 | 9 | 6 |
| 8A | 6 | 21 | 9 | 9 | 6 |
| 9A | 6 | 24 | 9 | 9 | 6 |
| 1B | 9 | 0 | 9 | 9 | 6 |
| 2B | 9 | 1 | 9 | 9 | 6 |
| 3B | 9 | 3 | 9 | 9 | 6 |
| 4B | 9 | 6 | 9 | 9 | 6 |
| 5B | 9 | 9 | 9 | 9 | 6 |
| 6B | 9 | 12 | 9 | 9 | 6 |
| 7B | 9 | 18 | 9 | 9 | 6 |
| 8B | 9 | 21 | 9 | 9 | 6 |
| 9B | 9 | 24 | 9 | 9 | 6 |
| 1C | 12 | 0 | 9 | 9 | 6 |
| 2C | 12 | 1 | 9 | 9 | 6 |
| 3C | 12 | 3 | 9 | 9 | 6 |
| 4C | 12 | 6 | 9 | 9 | 6 |
| 5C | 12 | 9 | 9 | 9 | 6 |
| 6C | 12 | 12 | 9 | 9 | 6 |
| 7C | 12 | 18 | 9 | 9 | 6 |
| 8C | 12 | 21 | 9 | 9 | 6 |
| 9C | 12 | 24 | 9 | 9 | 6 |
| 1X | 12 | 0 | 18 | 0 | 6 |
| 6X | 12 | 12 | 18 | 0 | 6 |
| 8X | 12 | 21 | 18 | 0 | 6 |
| 1Y | 12 | 0 | 0 | 18 | 6 |
| 6Y | 12 | 12 | 0 | 18 | 6 |
| 8Y | 12 | 21 | 0 | 18 | 6 |

No: Neem oil; Tw: Tween 80; Ne: Neem aqueous extract; Se: Sida weeds hydroalcoholic extract; Ca: Calcium extract.

The second formulation step was a 4-Factor mixture design (Simplex-Lattice design with two polynomial degree and reinforced with interior points and a centroid) selected based on data from the previous formulation stage. This 4-Factor mixture designs had 15 runs coded in Table 2 and the corresponding values of Tw, Ne, Se, No and Ca are shown in the Table 3. The stability of these mixtures was recorded after 48 h. The design of the matrix was carried out with the STATISTICA 10 software. The inhibition of the growth of *Fusarium oxysporum* was determined for each run at 20% (v/v) by the dilution method using PDA medium. A disc of fungi was introduced inside of the Petri dish, sealed and kept at 26 ± 2°C for 1 week. After that, the growth diameter was recorded, and the inhibition percentage was calculated using the formula below.

$$\%I=(Dc-De)*100/Dc$$

where Dc is the growth diameter of the negative control and De is the growth diameter of the medium with emulsion.

The biological activity of each emulsion was tested three time.

The modelling of stability and the biological activity of the second stage of formulation were carried out for the surface triangular representation of the data. Then, the linear, quadratic and special cubic model were analyzed for the stability model and the activity model as well as the best model was identified.

### *2.3. Evaluation of the Characteristics of the Best Bioformulated Emulsion Product*

#### *2.3.1. Evaluation of the Physico-Chemical Characteristics of the Best Bioformulated Emulsion Product*

The pH and conductivity of the stable emulsion were checked using a pH meter. The Thermodynamic stability was evaluated on the one hand by centrifugation and on the other hand by temperature variation. The developed formulation was subjected to centrifugation at 1, 000 ×g and 10, 000 ×g for 30 minutes at 25°C. Then, formulation mixture was observed for any phase separation [21]. The effect of temperature on the formulations was studied at 45°C and -20°C. For the effect of temperature at 45°C on the formulation, it was evaluated by incubation in a water bath at 45ºC for 6 h and at room temperature for 18 h while, the effect of a temperature of -20°C on the formulation was evaluated by exposing the emulsion to a cycle of -20ºC for 24 h and at room temperature for 24 h. This cycle was repeated three times, then each formulation was observed for the separation of the oil and/or cream phases [21]. The diameter of droplet of the stable emulsion with high biological activity was measured in photos taken on Neubauer hemocytometer with an optical microscope at magnifications of 400 and 1000.

***Table 2.*** *Coded of 4 factor Simplex lattice design for the fourth phase emulsion preparation from mixtures of Ne, Se, Ca, No and Tw.*

| Elements of mixtures | Mixture codes and proportions (mL) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| 1C | 1 | 0 | 0 | 0 | 0.5 | 0.5 | 0.5 | 0 | 0 | 0 | 0.625 | 0.125 | 0.125 | 0.125 | 0.25 |
| 8X | 0 | 1 | 0 | 0 | 0.5 | 0 | 0 | 0.5 | 0.5 | 0 | 0.125 | 0.625 | 0.125 | 0.125 | 0.25 |
| 8Y | 0 | 0 | 1 | 0 | 0 | 0.5 | 0 | 0.5 | 0 | 0.5 | 0.125 | 0.125 | 0.625 | 0.125 | 0.25 |
| 9C | 0 | 0 | 0 | 1 | 0 | 0 | 0.5 | 0 | 0.5 | 0.5 | 0.125 | 0.125 | 0.125 | 0.625 | 0.25 |

1C: content 9% Ne (m/V), 9% Se (m/V), 6% Ca (m/V), 12% No (V/V) and 0% Tw (V/V); 8X: content 9% Ne (m/V), 0% Se (m/V), 6% Ca (m/V), 12% No (V/V) and 21% Tw (V/V); 8Y: content 0% Ne (m/V), 9% Se (m/V), 6% Ca (m/V), 12% No (V/V) and 21% Tw (V/V); 9C: content 9% Ne (m/V), 9% Se (m/V), 6% Ca (m/V), 12% No (V/V) and 24% Tw (V/V); Ne: Neem aqueous extract; Se: Sida weeds hydroalcoholic extract; Ca: Calcium extract; No: Neem oil; Tw: Tween 80.

***Table 3.*** *Coded of the 4 factor Simplex lattice design and the corresponding amount of the extracts in the formulation Ne, Se, Ca, No and Tw.*

| **Mixture codes and proportions of the extracts (%)** | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Elements of the mixtures** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** |
| Tw (v/v) | 0 | 21 | 21 | 24 | 10.5 | 10.5 | 12 | 21 | 22.5 | 22.5 | 8.25 | 18.75 | 18.75 | 20.25 | 16.5 |
| Ne (w/v) | 9 | 18 | 0 | 9 | 13.5 | 4.5 | 9 | 9 | 13.5 | 4.5 | 9 | 13.5 | 13.5 | 13.5 | 9 |
| Se (w/v) | 9 | 0 | 18 | 9 | 4.5 | 13.5 | 9 | 9 | 4.5 | 13.5 | 9 | 13.5 | 13.5 | 13.5 | 9 |
| No (v/v) | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 |
| Ca (w/v) | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |

Tw: Tween 80; Ne: Neem aqueous extract; Se: Sida weeds hydroalcoholic extract; No: Neem oil; Ca: Calcium extract.

#### *2.3.2. Evaluation of the Biological Activity of the Best Bioformulated Emulsion Product*

The growth inhibitions of the best emulsion were obtained against fungi (*Fusarium oxysporum, Pythium myriotylum, Phytophthora infestans* and *Phytophthora megakarya*) by dilution method described previously using PDA medium incorporated with the best formulation at 20% and 10%; and against bacteria (*Ralstonia solanacearum*) using micro-plaque dilution to determine MIC. These assays were performed four times.

### *2.4. Evaluation of the Characteristics of the Best Bioformulated Emulsion Product After pH Correction*

#### *2.4.1. Production of the Best Bioformulated Emulsion Product with Corrected pH*

The correction of pH has been carried out considering the pH of the best formulation. To this end, a calcium oxide (CaO) was included in the process to produce the best formulation by replacing 0%, 15%, 16.67%, 25% or 33.33% (w/w) extract of calcium by CaO. Using the same process of the first and the second formulation step, the correction of pH induces the coalescence of emulsion. Then, the effect of formulation process on the coalescence was studied by comparing the three methodologies described below.

1) Every aqueous soluble extract was dissolved in water (aqueous phase or Aq) and Tween 80 (Tw) plus Neem oil (No) were added and vortexed for 1.5 to 2 minutes to form an emulsion. This abbreviated method "Aq+(Tw+No)" corresponds to the process used during the two formulation steps.
2) Aqueous phase (Aq) and Tween 80 (Tw) were added and vortexed for 0.75 to 1 min, then No was added and vortexed for 1.5 to 2 minutes to form emulsion. This method is the "(Aq+Tw) +No" process.
3) Tween 80 (Tw) plus No were vortexed for 0.75 to 1 min and each aqueous soluble extract dissolved in water was added and vortexed for 1.5 to 2 minutes to form an emulsion. This method is the "(Tw+No) +Aq" process.

The best process was used to prepare the formulation at the optimize pH, by a high-energy method which consist of mixing for 10 min to have emulsion with a chosen percentage of CaO.

#### *2.4.2. Evaluation of the Characteristics of the Corrected best Bioformulated Emulsion Product*

As the best formulation, the droplet size, pH, conductivity, thermodynamic stability and biological activity of the optimize formulation were evaluated. In addition, $MIC_{50}$ of the optimized formulation and the effect to the integrity of cell wall of *P. megakarya* were determined according to the protocol described by S. Limsuwan [22]. The effect of the optimize emulsion (1%), was evaluated on cocoa black pod disease (*Theobroma cacao* L., SCA12 × SNK413 variety) caused by *P. megakarya*, using detached leaf protocol describe by [23]. The experiment was performed two more times and the disease severity index was determined. Disease expression was assessed six days after inoculation, using the rating scale developed by [24, 25].

*Data analysis*

The results were expressed as means ± standard deviation and subjected to Analysis of Variance (ANOVA) and significant differences were established using the Tukey's test with $P$ value less than 0.05. STATISTICA 10 software was used to develop the validity of stability and activity models.

## 3. Results

### *3.1. Bioformulation of the Emulsion Product*

In the first step of bioformulation, the first group of the quasi-experiment model that had different concentrations of No and Tw had the maximum stability (100% stability) of the emulsion in the bioformulation having 6% No when 18% minimum of Tw was used (Figure 1). For the bioformulations with 9% and 12% of No, it is 21% and 24% of Tw which contribute to having the 100% of stability (Figure 1). Minimal stability (25% stability) was recorded in the surfactant-free bioformulation (Figure 1). By using 12% of No, the minimum stability is observed for the 1C emulsion and the maximum stability for the 9C emulsion. Both 1C and 9C emulsions were used in the simplex-Lattice design.

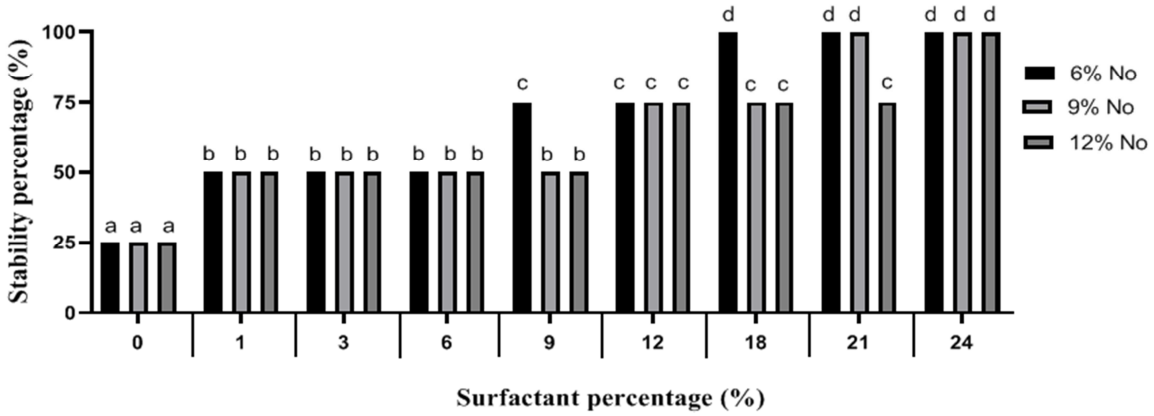



***Figure 1.*** *Stability of the emulsion in runs having Ne 9%, Se 9% and Ca 6% using the quasi-experiment design. No: Neem oil; Ne: Neem aqueous extract; Se: Sida hydroalcoholic extract; Ca: calcium extract. The means with different letters are different and significant at the 5% probability level.*

For the second group of this quasi-experiment where Se was absent and only Tw had a different concentration, we recorded 75% stability for the 8X trial which contains 12% of neem oil and 21% of surfactant after 48 h (Figure 2A). For the third group in this experiment where Ne was absent and only Tw had different concentration, the same percentage stability was recorded for the 8Y trial which contains 12% neem oil and 21% Tw after 48 h (Figure 2B). 8X and 8Y emulsions were also used in the simplex-Lattice design.

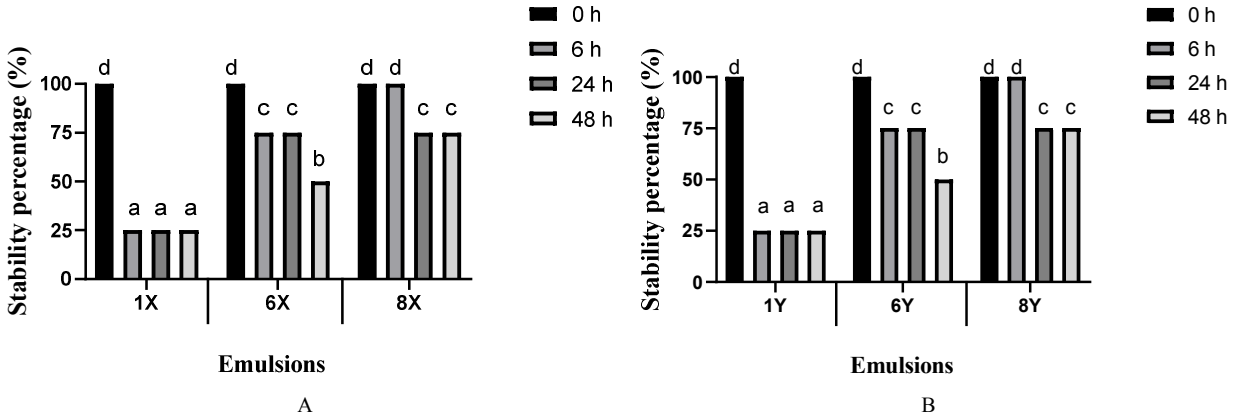



***Figure 2.*** *Stability of the emulsion using the quasi-experiment design. (A) Runs having Ne 9%, Se 0% and Ca 6% (runs 1X, 6X and 8X who respectively have 0%, 12% and 21% of NO). (B) Runs having Ne 0%, Se 9% and Ca 6% (runs 1Y, 6Y and 8Y who respectively have 0%, 12% and 21% of NO). No: Neem oil; Ne: Neem aqueous extract; Se: Sida hydroalcoholic extract; Ca: calcium extract. The means with different letters are different and significant at the 5% probability level.*

In the second step of bioformulation, regarding the stability of the fifteen mixtures (Figure 3A) and their model obtained from the 4 factors simplex-lattice design, the linear model was statistically significant with $P$= 0.000033 while quadratic and special cubic models were not significant with $P$= 0.712667 and $P$= 0.719258 respectively (Table 4). This linear model has confirmed the strong beneficial effect of the 9C emulsion on the stability model (Figure 4A). The stability equation obtained is presented below.

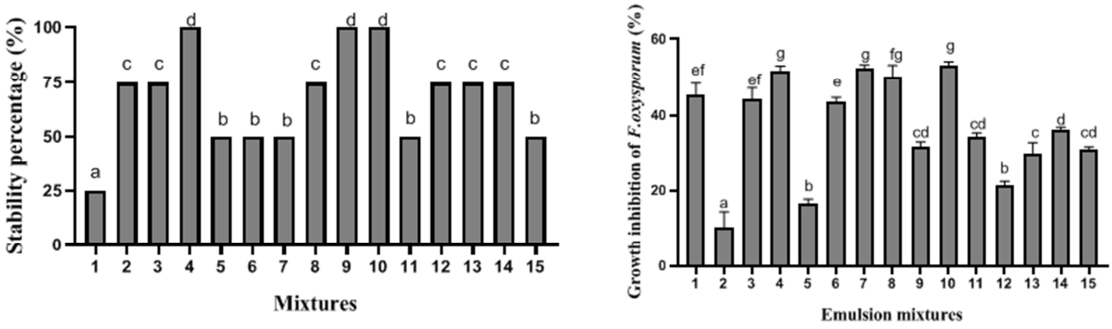
$$\%Stability = 20.119*1C + 77.262*8X + 77.262*8Y + 98.690*9C \quad (1)$$

where 1C, 8X, 8Y and 9C are the independent variables used for the construction of the Simplex-Lattice matrix.

Regarding the inhibition of the fifteen mixtures on *Fusarium oxysporum* (Figure 3B) and the activity model obtained from the 4 factors simplex-lattice design, the linear model had also shown the statistically significance with $P$ = 0.006820 while quadratic and special cubic model had $P$= 0.494183 and $P$= 0.554996 respectively (Table 4). This linear model showed that the 9C emulsion was the one with high beneficial effect on the activity model (Figure 4B). The best equation for % inhibition against *Fusarium oxysporum* was:

$$\%Inhibition_{Fusarium\ oxysporum} = 39.096*1C + 10.749*8X + 46.155*8Y + 50.301*9C \quad (2)$$

where 1C, 8X, 8Y and 9C are the independent variables used for the construction of the Simplex-Lattice matrix and only the 8X coefficient is not significant.

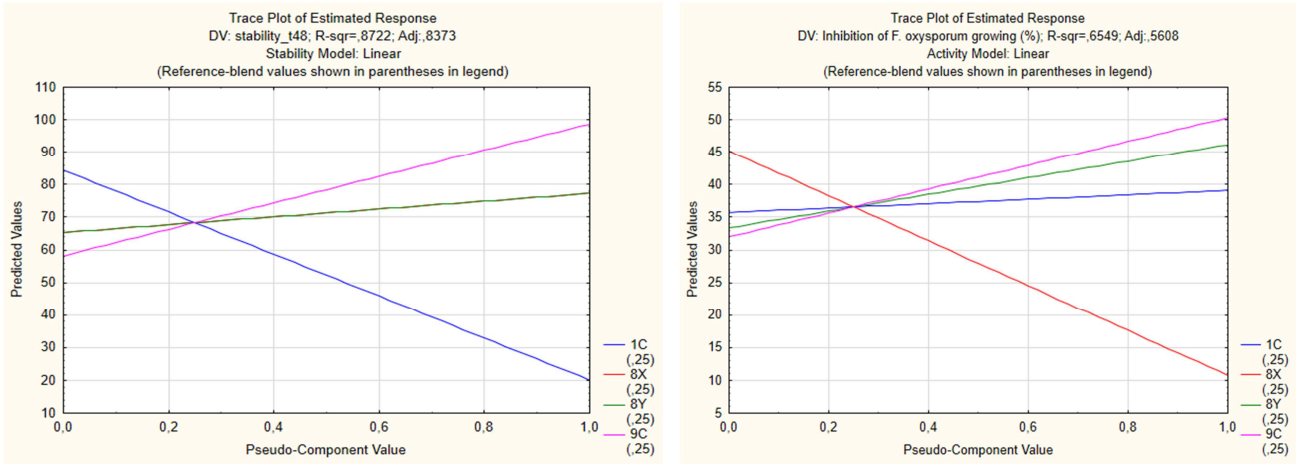



A B

***Figure 3.*** *Stability and activity against Fusarium oxysporum of the second stage of formulation using the simplex-lattice design. (A) Stability. (B) Activity. The means with different letters are different and significant at the 5% probability level.*

***Table 4.*** *Stability model and bioactivity model of the Simplex lattice coded design for the fourth phase emulsion.*

| | | | | | | |
|---|---|---|---|---|---|---|
| Parameters of the stability models | | | | | | |
| Model nature | F | P | R2 | AdjR2 | MS effect | MS error |
| Linear | 25.01704 | 0.000033 | 0.872169 | 0.837306 | 1986.607 | 79.4102 |
| Quadratic | 0.62007 | 0.712667 | 0.926706 | 0.794777 | 62.112 | 100.1684 |
| Special cubic | 0.64406 | 0.719258 | 0.979505 | 0.713076 | 90.199 | 140.0463 |
| Parameters of the linear stability model | | | | | | |
| Factor | 1C | 8X | 8Y | 9C | | |
| Coeff. | 20.11905 | 77.26190 | 77.26190 | 98.69048 | | |
| P | 0.008332 | 0.000001 | 0.000001 | 0.000001 | | |
| Cnf. Limt | [6.32; 33.92] | [63.46; 91.06] | [63.46; 91.06] | [84.89; 112.49] | | |
| Parameters of the bioactivity models: Fusarium oxysporum inhibition | | | | | | |
| Model nature | F | P | R2 | AdjR2 | MS effect | MS error |
| Linear | 6.959656 | 0.006820 | 0.654945 | 0.560839 | 556.2183 | 79.92038 |
| Quadratic | 1.037508 | 0.494183 | 0.846301 | 0.569644 | 81.2556 | 78.31808 |
| Special cubic | 1.395939 | 0.554996 | 0.976655 | 0.673168 | 83.0280 | 59.47826 |
| Parameters of the linear bioactivity model: Fusarium oxysporum inhibition | | | | | | |
| Factor | 1C | 8X | 8Y | 9C | | |
| Coeff. | 39.09617 | 10.74883 | 46.15500 | 50.30065 | | |
| P | 0.000066 | 0.115563 | 0.000015 | 0.000007 | | |
| Cnf. Limt | [25.25; 52.94] | [-3.1; 24.6] | [32.31; 60.00] | [36.45; 64.15] | | |




A B

***Figure 4.*** *Trace plot of estimated significant linear model response of the 4 factors of the simplex-lattice design use in the stage two formulation. 1C, 8X, 8Y and 9C are the independent factors. (A) Stability of the linear model. (B) Activity (growth inhibition of Fusarium oxysporum) linear model.*

### *3.2. Characterization of the Best Bioformulated Emulsion Product*

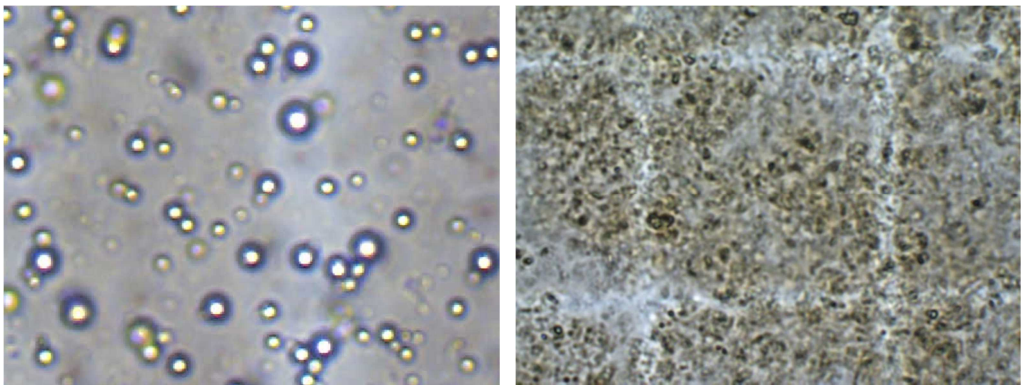
The best formulation was the 9C emulsion which is composed of water-soluble compounds (Ne 9%, Se 9%, Ca 6%), 12% (v/v) No and 24% Tw. This best formulation was very acidic despite surfactant concentration (Table 5). A pH of 2.84 and a conductivity 269 mV were recorded for the best formulation (Table 5). Table 5 also shows that centrifugation of the best bioformulation at 1000 ×g and 10000 ×g at 25°C

for 30 minutes decreases the stability by precipitating some compounds and changing the stable emulsion to one with precipitate and cream flocculation. At 45 °C, the best bioformulation is characterized by no precipitation but cream flocculation and oil (Table 5). The microscope image of the best bioformulation reveals the spherical shape of the emulsion, and also confirms the micro size of droplets (Figure 5). The maximum droplet size of the best recorded bioformulation was 9.72 µm (Table 5).

***Table 5.*** *Characterization of the best emulsion formulation and the optimize formulation.*

| Formulation | Shape | droplet size [µm] | PH | Conductivity [mV] | Centrifugation at 1000 ×g and 10,000 ×g | thermal stability at 45°C | thermal stability at -20°C |
|---|---|---|---|---|---|---|---|
| Best | Spherical | ≤ 9.72 | 2.84 (0.12) | 269.00 (18.00) | Precipitation and cream flocculation | Cream and oil flocculation | Precipitation and cream flocculation |
| Optimize | Invisible on the photonic microscope | Undetermined | 5.45 (0.18) | 81.24 (10.24) | Precipitation and no flocculation | Homogeneous | Homogeneous |

The values in the parenthesis are standard deviations.



A B

***Figure 5.*** *Microscopic Pictures of the best emulsions and the optimize emulsions at 1000 x magnification. (A) Best formulation. (B) Optimize formulation.*

The growth inhibition of the fungi *Fusarium oxysporum*, *Pythium myriotylum*, *Phytophthora infestans* and *Phytophthora megakarya* by the best bioformulation at 20% and 10% showed a dose-dependent activity (Table 6). These two formulations significantly decrease the growth from 7.90% to 55.25% when used at 20% (Table 6). In addition, the MIC of the best bioformulation against *Ralstonia solanacearum* was 80 µL/mL (Table 6).

***Table 6.*** *Biological activity of the best emulsion formulation and the optimize formulation.*

| | Concentration | | Growth inhibition (%) | | | |
|---|---|---|---|---|---|---|
| | [µL/mL] | [mg/mL] | Fusarium oxysporum | Pythium myriotylum | Phytophthora infestans | Phytophthora megakarya |
| Best emulsion | 200 | | 46.64 (4.77) cd | 55.25 (1.27) c | 48.6 (1.84) c | 39.2 (3.97) b |
| | 100 | | 23.16 (0.65) d | 7.90 (3.05) e | 24.36 (7.07) e | 20.69 (2.91) c |
| Optimize emulsion | 200 | | 72.48 (8.4) b | 83.46 (1.28) b | 87.8 (0.64) b | 100 (0.00) a |
| | 100 | | 48.03 (1.23) c | 35.03 (1.58) d | 37.4 (1.25) d | 100 (0.00) a |
| Negative Control | / | / | 0.00 (0.00) e | 0.00 (0.00) f | 0.00 (0.00) f | 0.00 (0.00) d |
| Positive control | / | 0.0033 | 100.00 (0.00) a | 100.00 (0.00) a | 100.00 (0.00) a | 100.00 (0.00) a |

The values in the parenthesis are standard deviations and the means in each column with different letters are different and significant at the 5% probability level.

### *3.3. Characteristics of the Best Bioformulated Emulsion Product After pH Correction*

By changing part of Ca to CaO, the pH also increases from 2.84 to 8.57 and the conductivity decreases from 269 mV to -107.8 mV (Figure 6). The use of pH adjusters at different concentrations with the Aq+(Tw+No) and (Aq+Tw)+No methods destabilizes the emulsion by a mechanism coalescence (characterized by the particles which become block at the interface and adopt a solid behavior) while (No+Tw)+Aq maintains stability. Then, the optimize formulation is composed of water-soluble compounds (Ne 9%, Se 9% Ca 4.5% and CaO 1.5% in w/v), 12% (v/v) No with 24% (v/v) Tw and the formulation method (No+Tw)+Aq.

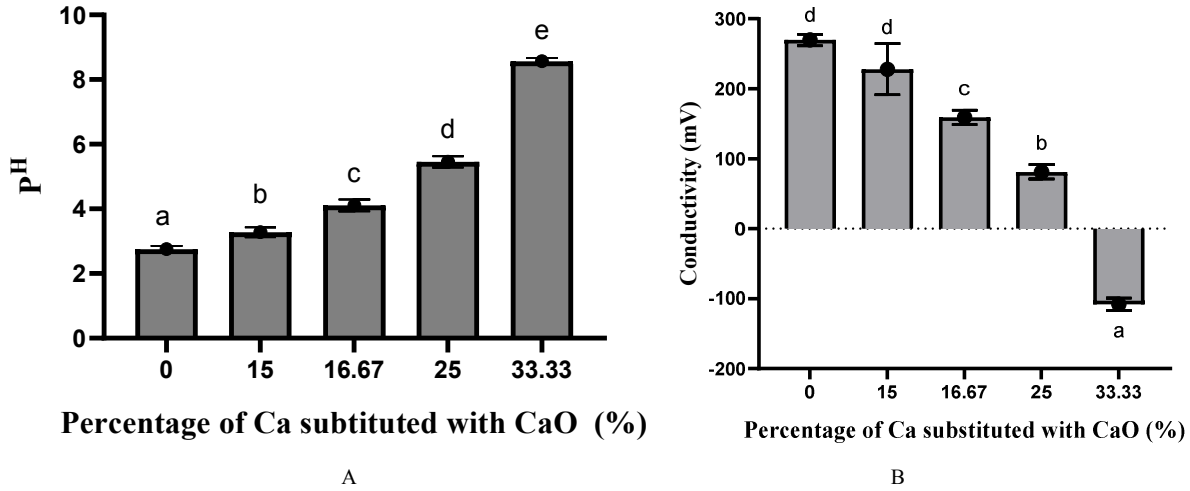



***Figure 6.*** *Effect of Calcium oxide on pH and Conductivity. (A) Effect of CaO on the pH. (B) Effect of CaO on the conductivity. The means with different letters are different and significant at the 5% probability level.*

The pH 5.45 and the conductivity 81.24 mV were recorded from the optimized bioformulation (Table 5). Table 5 also show that, centrifugation of the optimized bioformulation at 1000 ×g and 10,000 ×g at 25°C for 30 minutes decrease the stability by precipitating some compounds without cream flocculation. At 45°C thermal stability was observed for the optimized bioformulation without precipitation or flocculation (Table 5). The microscope image of the optimized bioformulation revealed that the droplets were imperceptible at 1000 magnificent (Figure 5B).

The inhibition of the growth of the fungi *Fusarium oxysporum*, *Pythium myriotylum*, *Phytophthora infestans* and *Phytophthora megakarya* by the optimized bioformulation at 20% and 10% showed a dose-dependent activity (Table 6). It significantly decreases growth from 72.48% to 100% when used at 20% (Table 6). The MIC of the formulation optimized against *Ralstonia solanacearum* was 9.6 µL/mL.

The optimized 1% bioformulation significantly reduced the growth of *Phytophthora megakarya* by 51.31% and reduced the formation of pathogenic cell wall by approximately 69% when used at 1% ($MIC_{50}$) and 2% ($2MIC_{50}$) after 24 h (Figure 7A and 7B) *in vitro*. In addition, the disease severity index obtained with detached leaf protocol inoculated with *Phytophthora megakarya* is significantly reduced *(P*< 0.05) from very susceptible to resistant with the disease severity index 4.2, 1 and 1.5 for negative control, positive control and optimize bioformulation respectively (Figure 7C).

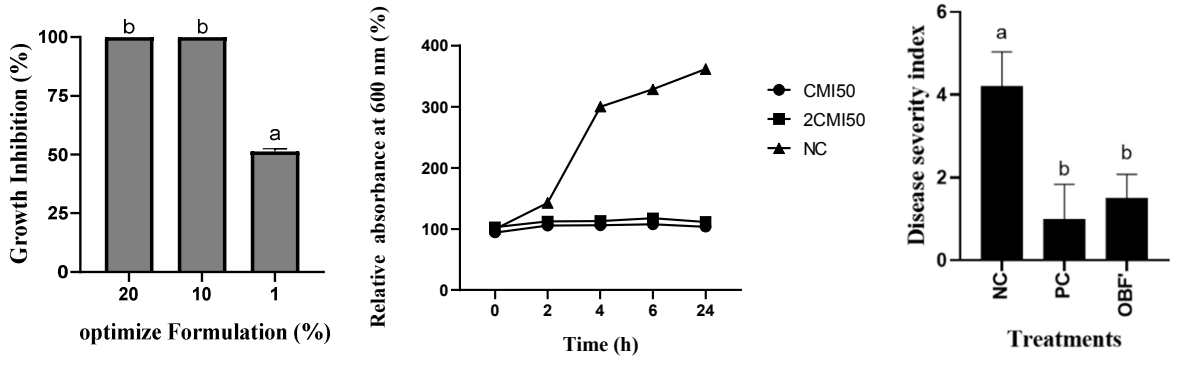



***Figure 7.*** *Effect of the optimize bioformulation on Phytophthora megakarya integrity and cocoa black pods disease severity. (A) Inhibition of the growing after a week. (B) Effect on the cell wall integrity. (C) Effect of treatments on cocoa black pods disease severity. The means with different letters are different and significant at the 5% probability level. NC: negative control, PC: positive control, OBF Optimized bioformulation.*

# 4. Discussion

Biopesticides are ecofriendly pesticides obtained from natural substances. They are more widely used in agriculture and health programs; and can be beneficial for the environment [26]. Research on biological control of cacao black pod disease in cacao production is sparse or in its infancy [27]. There is a steady increase in information on the influencing factors and mechanisms that underlie the biological control of cacao diseases as well as practical aspects such as production, formulation and application of inoculum. Intensive research on plant growth-promoting rhizobacteria (PGPR) or organic substances is carried out

worldwide to develop biofertilizers and biocontrol agents like actinobacteria *Streptomyces cameroonensis* or oyster shell powder amendment for cocoa protection against *P. megakarya* [4, 17]. Futher work on production, formulation is also needed for biocontrol to become economically attractive. There is a growing interest in formulations based on natural bioactive agents like plant extracts as they often break down quickly [28]. A new dynamic is trying to combine elicitors in formulations for agricultural purposes, as shown in [6].

This study presents the production of an optimized emulsion bioformulation based on No, Ne, Se, Ca and Tw that can be used for plant protection. The quasi-experimental design and the simplex-lattice showed that, increasing surfactant also increase stability while Ne and Se have the same effect on stability. By increasing Neem oil in a formulation with a fixed amount of surfactant, stability was decreased and activity was enhanced [29, 30]. In the present study, the stability of the emulsion can be justified by Tw, but also by the presence of saponins, polysaccharides and/or proteins in the Se extracts [31].

The biological activity of emulsions containing or not containing Se against *Fusarium oxysporum* shows that Ne and Se have an additive effect. This could be due to the nature of the compounds that constitute them and their properties. In fact, the presence of tannin, saponin, flavonoid, steroid, terpenoids, cardiac glycosides, cryptolepin and quindoline has been reported in *Sida acuta* extract, which may justify its activities [11, 31]. Neem extract has shown many bioactive properties such as antifungal [7, 32].

Correcting the pH from 2.84 to 5.45 increased the thermodynamic stability of the optimized formulations and their bioactivity. By mixing No and Tw and after adding the aqueous phase and mixing again using a high-energy (vortex 10 min), favored nano-emulsion characterized by fine droplet sizes which are not visible under microscope with 1000 x magnification. The stability of the optimized formulation corroborates those of [29] who managed to optimize Neem oil nano emulsion formulation with Tween 20 in distillated water by the high-energy method (30 to 45 min of sonication) and obtain smallest droplet size of 67.85 nm. These results also corroborate those of [33] who reported the *in vitro* effect of silicate hydroxide and calcium oxide at 1000 ppm on isolates of *F. oxysporum*, *A. alternata*, *A. niger*, *P. digitatum*, *R. stolonifer* and *P. italicum*. A possible mechanism of action could be associated with effects on virulence factors notably the enzyme protease.

The optimized emulsion showed a significant dose-dependent activity ($P< 0.05$) against *Phytophthora megakarya* with an inhibition of 51.31 % when the emulsions were used at 1% *in vitro* and a reduction in the severity index of disease from 4.2 to 1.5 when applied to cocoa leaves. The effect of the optimized emulsion on the severity of the infection may come from the inhibitory effect of the formulation on the pathogen and/or from the ability of calcium to increase plant defence. These results corroborate those obtained by [23, 34] who showed that pre-treatment of cocoa with calcium elicitors and snail shell powder (rich in calcium and chitins) respectively, reduced the severity index of the disease caused by *P. megakarya*.

# 5. Conclusion

The 4 Factor mixture designs, stability and activity models showed that the best formulation contains No 12% (v/v), Ne 9% (w/v), Se 9% (w/v), Ca 6% (w/v) and Tw 24% (v/v) with the "AQ + (Tw+No)" production process. Therefore, the optimized formulation contains No 12% (v/v), Ne 9% (w/v), Se 9% (w/v), Ca 4.5%(w/v), CaO 1.5% (w/v) and Tw 24% (v/v) with the "(Tw+No) + AQ" production process. The best formulation and the one optimized at room temperature have 100% stability and a significant dose-dependent activity ($P< 0.05$) against plant pathogens. We have 20.69% and 100% inhibition against *Phytophthora megakarya* with the best emulsion and the one optimized respectively at 10%. When the optimized formulation is applied at 1% on the detached cocoa leaves before inoculation with *Phytophthora megakarya*, it reduces the disease severity index from 4.2 to 1.5. These results suggest that our models and bioformulation could be useful for protection of cocoa against *P. megakarya*, the causal agent of Black Pod Disease.

# Disclosure Statement

The authors declare no conflicts of interest regarding the publication of this paper.

# Acknowledgments

The authors acknowledge the Biotechnology Centre of the University of Yaoundé 1 for allowing laboratory facility to conduct this study. The authors are also grateful to Victorine Yaya FORNKWA and Romaric Armel MOUAFO TCHINDA for their writing assistance and proof-reading assistance.

# References

[1] G. Lescuyer, L. Boutinot, P. Goglio et al., "Analyse de la chaîne de valeur du cacao au Cameroun," Rapport pour l'Union Européenne, DG DEVCO. Value Chain Analysis for Development Project (VCA4D CTR 2016/375-804)," p. 127, 2020.

[2] M. Ndoumbe-Nkeng, C. Cilas, E. Nyemb et al., "Impact of Removing Diseased Pods on Cacao Black Pod Caused by *Phytophthora megakarya* and on Cacao Production in Cameroon," *Journal of Crop Protection.* vol. 23, pp. 415–424, 2004.

[3] J. B. Mbarga, G. M. T. Hoopen, J. Kuate et al., "*Trichoderma Asperellum*: A potential biocontrol agent for *Pythium myriotylum* causal agent of cocoyam (*Xanthosoma sagittifolium*) root rot disease in Cameroon," Crop Protection, vol. 36, pp. 18–22, 2012.

[4] P. M. Tene-Tayo, A. Dzelamonyuy, D. Omokolo Ndoumou et al., "Enhancement of *Theobroma cacao* seedling growth and tolerance to *Phytophthora megakarya* by heat-treated oyster shell powder," American Journal of Plant Sciences, vol. 10, pp. 578-594, 2019.

[5] A. Dzelamonyuy, P. M. Tene-Tayo, E. R. Ngotcho et al., "Development of a powder formulation of *Streptomyces cameroonensis* and evaluation of its biocontrol and growth promoting activities against *Phytophthora megakarya* (causal agent of black pod disease) for two cocoa hybrids from Cameroon," Journal of Microbiology and Biotechnology, vol. 32 no. 2, pp. 160-169, 2022.

[6] Guomin Yang, Yanling Yao & Na Li. (2022). Knowledge, Attitudes and Practices (KAP) Related to the Coronavirus Disease 2019 (COVID-19) in a Village in Southern China: A Cross-sectional Survey. *World Journal of Public Health* (1). doi: 10.11648/J.WJPH.20220701.14 A. Jamiołkowska, "Natural Compounds as elicitors of plant resistance against diseases and new biocontrol strategies," Agronomy, vol. 10, no. 2, p 173, 2020.

[7] K. Biswas, I. Chattopadhyay, R. K. Banerjee et al., "Biological activities and medicinal properties of neem (*Azadirachta indica*)," Current science, vol. 82, no. 11, pp 1336-1345, 2002.

[8] C. Wanren and L. Hua, "A brief review of the extraction technologies of azadirachtin, a plant-derived bio-pesticide," African Journal of Engineering Research, vol. 6, no. 1, pp. 15-8, 2018.

[9] S. Mohideen, E. Sasikala and V. Gopal, "Pharmacognostic studies on *Sida acuta* Burm. f.," Ancient science of life, vol. 22, no. 1, pp. 57-66, 2002.

[10] O. T. Tcheghebe, A. J. Seukep and F. N. Tatong, "Ethnomedicinal uses, phytochemical and pharmacological profiles, and toxicity of *Sida acuta* Burm. f.," The Pharma Innovation Journal. vol. 6, no. 6, pp 01-06, 2017.

[11] D. Karou, A. Savadogo, A. Canini et al. "Antibacterial activity of alkaloids from *Sida acuta*," African Journal of biotechnology. vol. 4, no. 12, pp. 1452-7, 2005.

[12] I. R. Iroha, E. S. Amadi, A. C. Nwuzo et al., "Evaluation of the antimicrobial avtivity of extracts of *S. acuta* against clinical isolates of *Staphylococcus aueus* isolated from human immunodeficiency virus/acquired immunodeficiency syndrome patients," Research Journal of Pharmacology. Vol. 3, no. 2, pp. 22-25, 2009.

[13] C. Nisha, P. Bhawana and M. H. Fulekar, "Antimicrobial Potential of Green Synthesized Silver nanoparticles using *Sida acuta* leaf extract," Nano Science and Nano Technology. Vol. 11, no. 1, pp. 111-119, 2017.

[14] B. Maia, S. Arcaro, M. Souza et al., "Characterization of sand casting and oyster shells as potential sources of raw material to produce soda-lime glasses," Chemical Engineering and Transformation Journal, vol. 43, pp. 1795-1800, 2015.

[15] N. Prabhakar and S. R. N. P. Jayashankar, "Relevance of Calcium Nutrition in Present Day Agriculture technology refinement for biochar production and evaluation of its effect on soil health and crop productivity view project waste management view project," Advances in Life Sciences, vol. 5, pp. 2526–2530, 2016.

[16] A. S. Djabou-Mouafi, Y. Qin, T. Boudjeko et al. "Effects of calcium and magnesium fertilization on antioxidant 2 activities during cassava postharvest physiological deterioration," Crop Science, vol. 58, pp. 1–8, 2018.

[17] S. E. Ranf, P. Lennart, J. Pascal et al., "Interplay between calcium signalling and early signalling elements during defence responses to microbe- or damage-associated molecular patterns," Plant Journal, vol. 68, pp. 100–113., 2011.

[18] F. Lesueur, "Élaboration de formulations à base d'extraits de neem (*Azadirachta indica* A. Juss) pour la protection de la pomme de terre (*Solanum tuberosum* L.) contre le *Myzus persicae*, un puceron colonisateur et vecteur de virus circulants et non circulants," p 139. 2006.

[19] J. R. Kuiate, K. M. L. Brigitte, R. K. Jules et al., "Screening of Some Plants Used in the Cameroonian Folk Medicine for the Treatment of Infectious Diseases," International Journal of Biology, vol. 3, no. 4, pp. 13-21.

[20] M. Gaonkar and A. C. Innovative, "Application of eggshell as fertilizer and calcium supplement tablet," International Journal of Innovative Research in Science, Engineering and Technology, vol. 5, no. 3, pp. 3520–5, 2016.

[21] J. Nelson, T. E. Y. S. Johnson, P. S. Vichangal et al., "Formulation of sweet flag oil (*Acorus calamus*) nanoemulsion by spontaneous emulsification method for the management of *Sitophilus oryzae*," International Journal of Chemical Studies, vol. 7, no. 3, pp. 2072–6, 2019.

[22] S. Limsuwan and S. P. Voravuthikunchai, "Bactericidal, Bacteriolytic, and Antibacterial Virulence Activities of *Boesenbergia pandurata* (Roxb) Schltr Extract against *Streptococcus pyogenes*," Tropical Journal of Pharmaceutical Research, vol. 12, no. 6, pp. 1023–8, 2013.

[23] Y. Zhang, P. Smith, S. N. Maximova et al., "Application of glycerol as a foliar spray activates the defense response and enhances disease resistance of *Theobroma cacao*," Molecular Plant Pathology, vol. 16, no. 1, pp. 27–37, 2015.

[24] S. Nyassé, C. Cilas, C. Herail et al., "Leaf inoculation as an early screening test for cocoa (*Theobroma cacao* L.) resistance to *Phytophthora black* pod disease," Crop Protection, vol. 14, no. 8, pp; 657–63, 1995.

[25] D. Paulin, M. Ducamp and P. Lachenaud, "New sources of resistance to *Phytophthora megakarya* identified in wild cocoa tree populations of French Guiana," Crop Protection, vol. 27, no. 7, pp. 1143–7. 2008.

[26] D. Srijita, "Biopesticides: an ecofriendly approach for pest control, "World Journal of Pharmacy and Pharmaceutical Sciences, vol. 4, no. 6, pp. 250–65, 2015.

[27] G. M. Ten Hoopen and U. Krauss, "Biological Control of Cocoa Disease," Springer, Cham, pp 511-566. 2016.

[28] R. S. Ghotbi, M. Khatibzadeh and S. Kordbacheh, "Preparation of Neem Seed Oil Nanoemulsion," In: Proceedings of the 5 th International Conference on Nanotechnology: Fundamentals and Applications, Pp 11-13, 2014.

[29] M. Bhattacharya and S. Dixit, "Emulsification of Neem oil: effect of polar solvents and chain length of surfactant," *International Journal of chemical and pharmaceutical sciences.* vol. 3, no. 12, pp. 2175–9, 2015.

[30] K. R. S. Namasivayam, F. Lazar, H. Sanjana et al. "Effect of polymer-based surface coating on droplet size and potential pharmacological properties of neem oil," *IOAB Journal,* vol. 8, pp. 1–6, 2017.

[31] P. Nwankpa, O. Chukwuemeka, G. Uloneme et al. "Phyto-nutrient composition and antioxidative potential of ethanolic leaf extract of *Sida acuta* in wistar albino rats," African Journal of Biotechnology, vol. 14, no. 49, pp. 3264–9, 2015.

[32] J. Pohe and T. Agneroh, "L'huile des graines de neem, un fongicide alternatif à l'oxyde de cuivre dans la lutte contre la pourriture brune des cabosses de cacaoyer en Côte d'Ivoire," *Journal of Applied Biosciences*, vol. 62, pp. 4644–4652, 2013.

[33] A. Khaled and R. Mohamed, "Effet de composés calciques inorganiques sur le développement *in vitro* de moisissures isolées d'agrumes après la récolte," *Bulletin de la Société Royale des Sciences de Liège*, vol. 85, pp. 263–75, 2016.

[34] P. M. Téné-Tayo, C. A. Ewane, P. O. Effa et al., "Effects of chitosan and snail shell powder on cocoa (*Theobroma cacao* L.) growth and resistance against black pod disease caused by *Phytophthora megakarya*," African journal of Plant Science, vol. 11, no. 8, pp. 331-340, 2017.