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

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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 $26 \pm 2°C$ 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 -20°C 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.
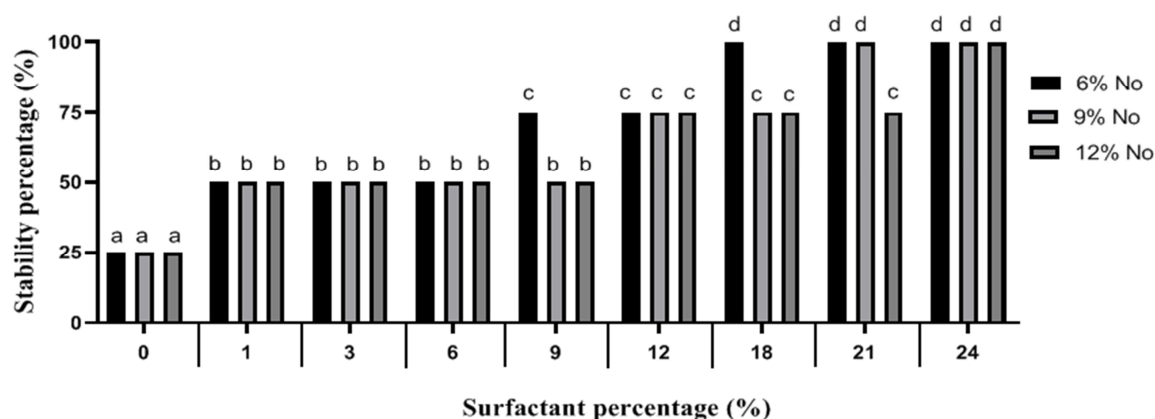
Figure 1. *Stability of the emulsion in runs having Ne 9%, Se 9% and Ca 6% using the quasi-experiment design. No: Neem oil; Ne: Neem aqueous extract; Se: Sida hydroalcoholic extract; Ca: calcium extract. The means with different letters are different and significant at the 5% probability level.*

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.
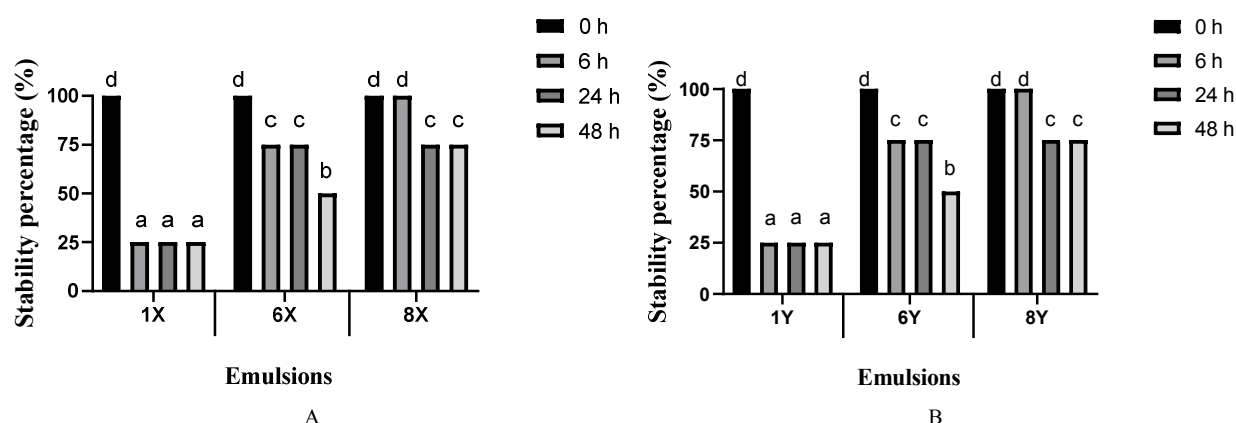

Figure 2. *Stability of the emulsion using the quasi-experiment design. (A) Runs having Ne 9%, Se 0% and Ca 6% (runs 1X, 6X and 8X who respectively have 0%, 12% and 21% of NO). (B) Runs having Ne 0%, Se 9% and Ca 6% (runs 1Y, 6Y and 8Y who respectively have 0%, 12% and 21% of NO). No: Neem oil; Ne: Neem 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.

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

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:

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

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.
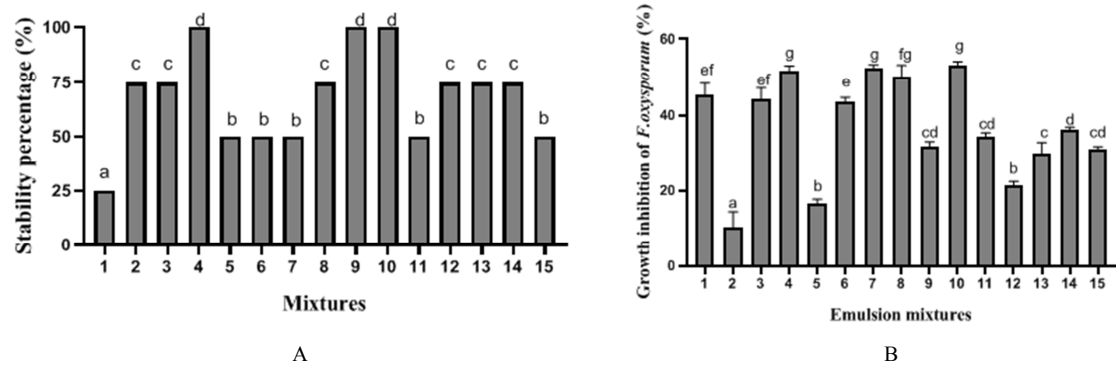
A

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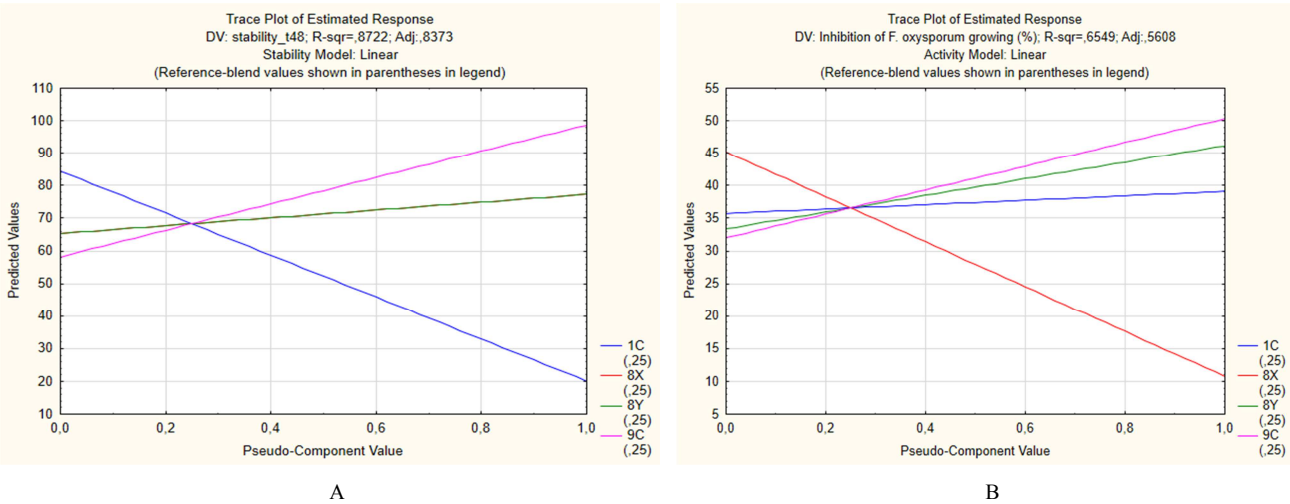
Figure 4. *Trace plot of estimated significant linear model response of the 4 factors of the simplex-lattice design use in the stage two formulation. 1C, 8X, 8Y and 9C are the independent factors. (A) Stability of the linear model. (B) Activity (growth inhibition of Fusarium oxysporum) linear model.*

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

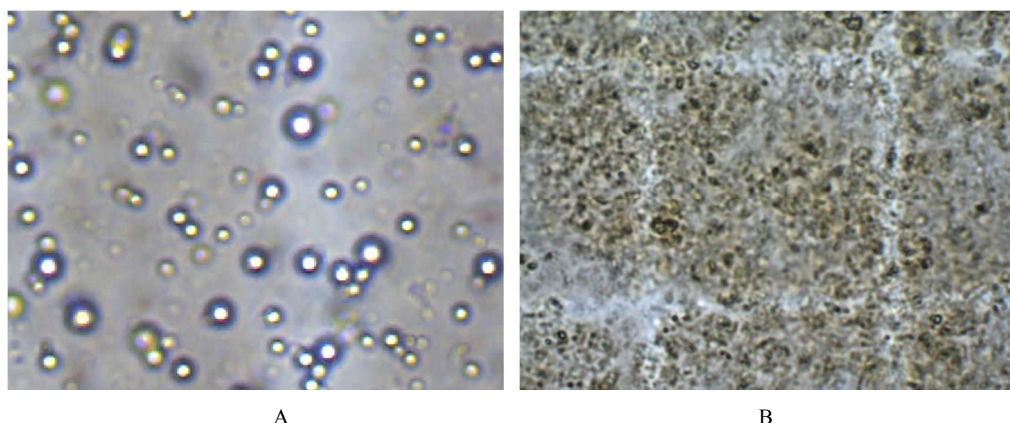The values in the parenthesis are standard deviations.

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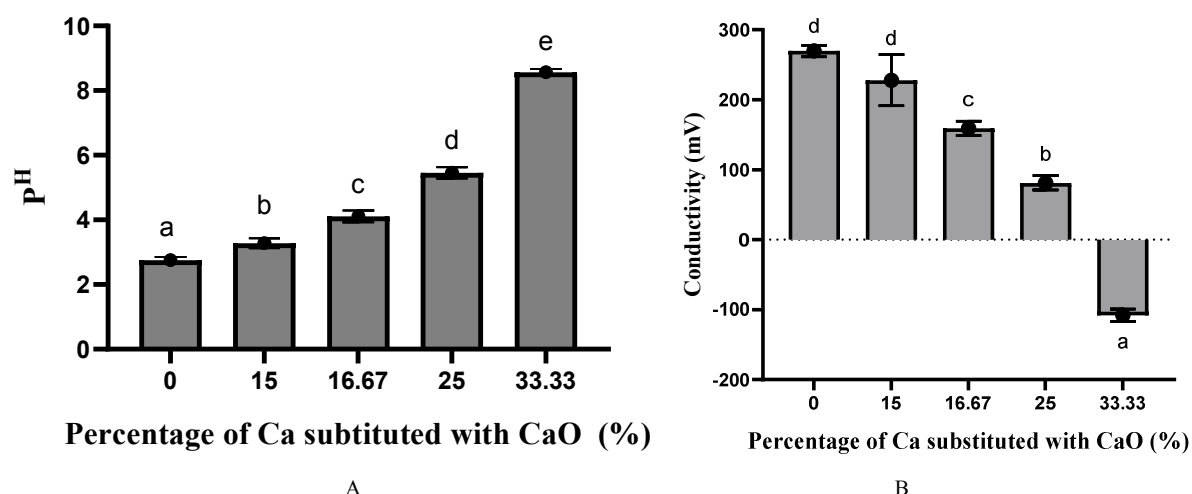
Figure 6. *Effect of Calcium oxide on pH and Conductivity. (A) Effect of CaO on the pH. (B) Effect of CaO on the conductivity. The means with different letters are different and significant at the 5% probability level.*

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).
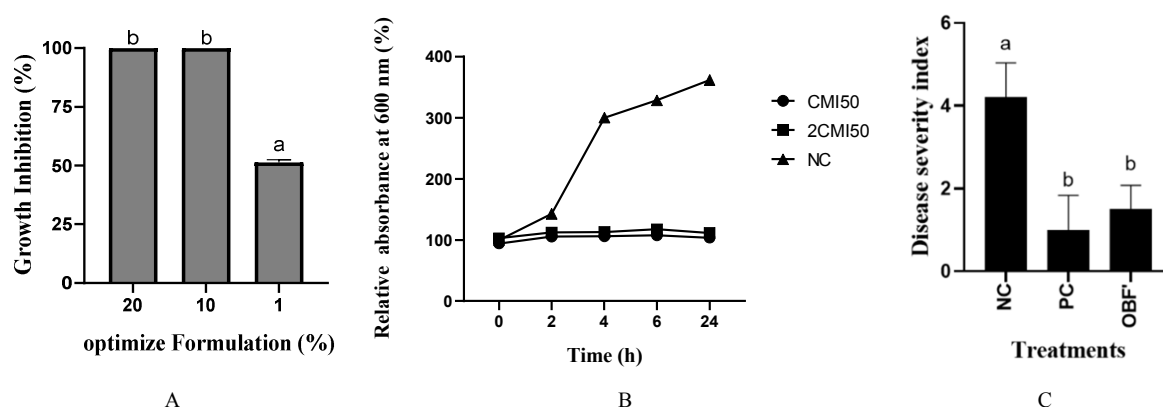

Figure 7. *Effect of the optimize bioformulation on Phytophthora megakarya integrity and cocoa black pods disease severity. (A) Inhibition of the growing after a week. (B) Effect on the cell wall integrity. (C) Effect of treatments on cocoa black pods disease severity. The means with different letters 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

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