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Formulation of Biofungicides from *Cymbopogon citratus* and *Tithonia diversifolia*: Evaluating Its Antimicrobial Activities against *Pythium myriotylum*, the Causal Agent of Root Rot of *Xanthosoma sagittifolium* (L.) Schott

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Abstract

Three fungicide formulations, namely M1, M2 and M3, were prepared from sodium bicarbonate, citronella essential oil and sunflower slurry. The stability of M1, M2 and M3 formulations was determined based on pH, temperature, order of incorporation of the inputs and storage time. The most stable formulations were used for antagonistic tests on *Pythium myriotylum*. The Minimum Inhibitory Concentration (MIC) was used for the greenhouse tests and the mode of action was determined *in vitro*. The study showed that the order of incorporation of the inputs "Essential Oil-Tween 80-Bicarbonate-Slurry" (EO-T80-B-S) promotes stability. M1 and M2 are stable at 4˚C, 25˚C, 37˚C and 40˚C temperatures and have a pH of 7 and 8 respectively. The Minimum Inhibitory Concentration of M1 and M2 is 1% on *P. myriotylum*. M1 and M2 act on Pythium by membrane lysis, inhibiting proton pumps and inhibiting protein synthesis. The formulations M1 and M2 reduce the incidence of root rot disease in cocoyam plants growth in the greenhouse. M1 and M2 are potential candidates for improvement of cocoyam seedlings production in Cameroon.

Keywords

Biofungicide, *Xanthosoma sagittifolium*, *Pythium myriotylum*, Root Rot Disease

1. Introduction

Cocoyam (*Xanthosoma sagittifolium* L. Schott) is a staple food in the tropics and subtropics and is one of the six most important root and tuber crops worldwide. Africa, as a continent, produces more than 71% of the world's cocoyam [1]. In Cameroon, it is the second most consumed tuber crop after cassava. Its edible tubers and young leaves contain carbohydrates and essential amounts of proteins, vitamins, and minerals and it provides cash income for farmers [2]. Root rot disease caused by *Pythium myriotylum*, is a major disease that limits the growth and productivity of this crop in Cameroon [3]. *P. myriotylum* is the most devasting disease of cocoyam and it is responsible for up to 90% loss of yield in some plantation. Although the root rot disease and the causal pathogen have been well characterized, knowledge of their reliable control measures is still poor [4] [5] [6]. Additionally, no resistant cocoyam variety has yet been developed. In general, control of *P. myriotylum* consists of the use of chemical fungicide. The multiplication of applications of these molecules poses serious problems of persistence [7]. Most of the current synthetic fungicides directly affect *P. myriotylum* essential functions, such as respiration, sterol biosynthesis or cell division [8]. This type of mode of action on one hand can lead to risks for humans and non-target organisms and on other hand to the development of resistant fungal strains. Therefore, the search for alternative ecofriendly tools to chemical fungicides is necessary. The judicious use of the local plant extracts as natural pesticides is an interesting alternative to protect crops, the environment and living organisms [9] [10]. These natural plant-based pesticides have already been the subject of several studies [11]. In addition, the antifungal properties of some plants have been proven. Among these plants, *Cymbopogon citratus* (citronella) is a pesticidal plant inhibiting several fungal pathogens and considered as an effective biocontrol agent. Therefore, citronella extract and essential oil have been found to mainly contain geraniol, citronellal and citronellol which are the monoterpenes' compounds [12]. Likewise, *Tithonia diversifolia* extracts which are recognized as a systemic pesticide have been found to contain alkaloids, tannins and flavonoids which are bioactive compounds that possess antimicrobial activity [13] [14]. A synergistic action of these pesticidal plants could lead to total protection of the plant because it would act by contact (*C. citratus*) and in a systemic way (*T. diversifolia*), hence a formulation based on *C. citratus* and *T. diversifolia*. Many plants based biopesticides which have been formulated through the mixture of several tropical plant extracts have been shown to increase the level of seedling protection and enhanced the expression of plant defense meta-

bololites during plant/pathogens interactions [15]. The objective of this work is to determine the efficacy and mode of action of the formulations prepared based on citronella essential oil and sunflower slurry against *P. myriotylum* as well as protection of cocoyam seedlings.

2. Material and Methods

2.1. Plants Material and Soil

The cocoyam seeds were obtained from Nkolbisson (Centre region, Cameroon) and were harvested in June, a rainy month. The varieties used are the white and red cultivars. The soil used to grow the cocoyam seedlings was black humus soil, often used by cocoyam seedling producers in the environs of Yaounde, Centre Region, Cameroon. The soil collected was air dried, sieved through a 10 mm sieve to get rid of hard material and debris, and then mixed with river sand in the ratio 3:1 w/w.

2.2. Pathogenic Fungi

Pythium myriotylum, *Phytophthora megakarya*, *Fusarium oxysporum*, *Phytophtora colocasia*, *Phytophtora infestans* strains used in this study was obtained from the microorganism bank of the Laboratory of Phytoprotection and Valorisation of Genetic Ressources (LPVGR), Biotechnology Centre, University of Yaounde 1, Cameroon. The strains were activited on Potato-Dextrose Agar (PDA) and incubated at 26˚C. Zoospore suspensions of pathogenic fungi were obtained as described by [1].

2.3. Preparation of *T. diversifolia* Extract and Citronella Essential Oil

T. diversifolia leaves was collected around the Biotechnology Centre of the University of Yaounde 1, Cameroon. The leaves were washed in running tap water, cut and then mixed with water in the ratio of 1:5 (W/V) before fermentation in bucket for 15 days according to the method described by [14]. At the end of the fermentation period, the slurry juice obtained was stored at 4˚C for futher use. The essential oil of citronella was obtained by hydrodistillation according to the method described by [16]. Briefly, 500 g of fresh citronella leaves and stems, collected at Nkolbisson were introduced into a water bath and the mixture was subjected to hydrodistillation for two hours using a clevenger-type apparatus. The essential oil collected by decantation at the end of the distillation was filtered on an anhydrous sodium sulphate column to remove any residual water. The resulting essential oil was stored in dark bottles at 4˚C. The extraction yield was expressed as a percentage and calculated according to the formula:

$$\%\ \text{Yield} = \frac{\text{VEO}}{\text{MF}} \times 100$$

with % Yield: percentage yield of essential oil; VEO: Volume of essential oil (ml); MF: Mass of fresh plant material (g).

2.4. Preparation of Different Formulations Tests

Three formulations namely M1, M2 and M3 were prepared with citronella essential oil, sodium bicarbonate, Tween 80 and sunflowers slurry according to the Table 1.

2.5. Physico-Chemical Characterization and Stability of the Emulsions

The stability of the emulsions depended on the size of the micelles, the order of administration of the inputs, the homogeneity, the pH, the temperature, the bicarbonate concentration and the time of conservation. The order of incorporation of the ingredients was evaluated using the following combinations: 1-2-3-4, 4-3-2-1 and 3-1-4-2 (Table 1).

Physical characteristics including color, odor, the presence of precipitates and appearance of the prepared emulsions were determined according to method described by [17]. The color was observed by visualization and odor determined; the stability of the formulations was determined at different temperatures during 180 days (4˚C, 25˚C, 37˚C and 40˚C), the size of the micelles assessed using an optical microscope and the pH determined using a pH meter

2.6. Determination of Antifungal Activity and Minimum Inhibitory Concentration

The efficacy of the stable formulation was evaluated *In vitro* against four oomycete (*P. myriotylum*, *P. infestans*, *P. colocaceae* and *P. megakarya*) and one true fungy (*F. oxysporum*). This was done using the agar well diffusion method. PDA medium was prepared and supplemented with the formulations at various concentrations (10% v/v, 5% v/v, 1% v/v, 0.1% v/v, 0.01% v/v) and chemical fungicide (Plantomil 72 WP with active compound metalaxyl and copper oxide) at 0.33 w/v % as recommended by the manufacturer. *P. myriotylum* and other target pathogenic microorganisms were cultured in the middle of the solid PDA medium and the percentage radial growth inhibition was calculated after 7 days using the formula:

$$\text{Inhibition}(\%) = \frac{\text{Radial growth of control} - \text{Radial growth of treatment}}{\text{Radial growth of control}} \times 100$$

Table 1. Composition of M1, M2 and M3 formulations.

Formulations inputs	Number corresponding to inputs	Formulations		
		M1	M2	M3
C. citratus essential oil	1	1%	1%	1%
Tween 80	2	0.5%	0.5%	0.5%
Sodium bicarbonate	3	5%	10%	15%
Tithonia diversifolia fermented extract	4	93.5%	88.5%	83.5%

This test was performed in triplicates. The 1%, 0.1% and 0.01% concentrations of the M1 and M2 formulations were used for the determination of the fungicidal or fungistatic activity [18].

2.7. Mode of Action of M1 and M2 Formulations

Measurement of the lytic activity of the formulations

The modified protocol of [19] was used to conduct this experiment. For this purpose, a standardised fungal suspension at the 0.5 McFarland scale in 0.9% NaCl was prepared. A volume of 100 µl of formulation was introduced into different tubes containing this suspension in order to have concentrations of the formulations equivalent to Minimum Inhibitory Concentration (MIC) and two minimum inhibitory concentration (2 MIC) in the medium. The suspensions obtained were incubated at 37˚C while stirring. At times 0 h, 2 h, 4 h and 6 h, the absorbance was measured at 620 nm. The absorbance at zero-hour (0 h) was used to evaluate the relative absorbance (Ar) at different times in order to draw the Ar = f (t) curve.

Effect of the formulations on protein synthesis

In five tubes each containing 9 ml of suspension PDB (potato dextrose broth), 0.5 ml of fungal suspension (standardised to McFarland's 0.5 scale) was added to each tube. The formulation was then added to each preceding mixture to give the concentrations: 2 MIC, MIC and 1/2 MIC. The control tube was treated under the same conditions and received 0.5 ml of PDB instead of the formulation. The tubes were incubated at 37˚C with a rotation of 80 g. After 24 hours of incubation, centrifugation at 5000 g for 2 minutes was used to recover the fungal cells, which were weighed and mixed with the lysis buffer at a rate of 40 mg of fungi per 500 ml of buffer. After 1 hour of incubation, a centrifugation at 10,000 g for 3 minutes allowed the recovery of the supernatant containing the proteins, which were then assayed by the Bradford reagent using the microplate reader (FLUOstar Omega Microplate Reader). The lysis buffer constituted the blank [20].

Effect of the formulations on the inhibition of ATPase /H+ proton pumps

The evaluation of the inhibitory effect of the formulations on proton pumps was carried out by controlling the pH of the spore suspension medium according to the protocol described by [21]. Inhibition of the acidification of the medium in the presence of the formulation was attributed to an inhibitory effect of the functioning of the H+ ATPase pumps by the formulation. 4 ml for the sporal solution was prepared and 0.5 ml of the formulation was added to obtain formulation concentrations equal to 1/2 MIC, MIC and 2 MIC. After 10 min of pre-incubation at 37˚C, acidification of the medium was initiated by adding 0.5 ml of 20% glucose solution whose rapid catabolism will be accompanied by the release of protons into the medium. Thereafter, the pH of the medium was measured every 15 min for 1 h 30 min. For the negative control, the extract was replaced by water; the pH values noted made it possible to draw the curve of pH variation as a function of time.

2.8. Production of Cocoyam Plant Seedlings under Greenhouse Conditions

The planting material consisted of white and red cultivars of *X. sagittifolium* minifragments of mean weight 18 g produced from minifragmentation techniques according to method described by [22]. The treated minifragments cocoyam seedling was then planted in pots containing soil and river sand mixed in the ratio 3:1. Each treatment was in duplicates of 10 seeds. The pots were kept in a greenhouse and watered with distilled water every two days for a period of 24 weeks.

The formulations were assessed for its efficacy in controlling the incidence of *P. myriotylum* through artificial soil inoculation with *P. myriotylum* zoospores according to [23]. Briefly, three months after cocoyam seedlings growth, the soil were artificially infested with 50 µl *P. myriotylum* zoospores calibrated at 106 zoospore/ml. Disease incidence was determinate two weeks after inoculation. Symptoms appearing on the leaves were described according to scoring scale by [23]. This score varies from 0 to 4 and is defined as follows: 0 = no disease symptoms on the leaves (0%); 1 = early yellowing of the leaves (1% - 25%); 2 = total and pronounced yellowing of the whole leaf (26% - 50%); 3 = early drying of the leaf (51% - 75%); 4 = total drying and death of the whole leaf (76% - 100%). Disease incidence was calculated as follow:

$$\mathrm{DI} = \frac{\left(\left(1\times n1\right)+\left(2\times n2\right)+\left(3\times n3\right)+\left(4\times n4\right)\right)}{\left(4\times N\right)}\times 100$$

With DI: disease incidence; *N*: the total number of plants; *n*1: the number of plants scoring 1; *n*2: the number of plants scoring 2; *n*3: the number of plants scoring 3; *n*4: the number of plants scoring 4.

2.9. Statistical Analysis

All experiments were conducted in triplicates and all data was expressed as means ± standard deviation and subjected to One-Way ANOVA. Tukey's test and probability values of $P \leq 0.05$ were considered to be significant using the GraphPad Prism software version 8.0.1.

3. Results

3.1. Evaluation of the Stability of the Different Formulations

Stability according to the order of incorporation of the inputs

With regards to the evaluation of stability as a function of the order of administration of the inputs over time, it appears that the order of administration: Essential Oil (EO)-Tween 80 (T80)-Bicarbonate (B)-Slurry (S) (EO-T80-B-S) favors the stability of the mixture compared to the order Slurry (S)-Bicarbonate (B)-Essential Oil-Tween 80 (T80) (S-B-EO-T80) and the order Bicarbonate (B)-Essential Oil (EO)-Slurry (S)-Tween80 (T80) (B-EO-S-T80). Indeed, the order EO-T80-B-S has a stability score that remains high (score = 4) after 180 days

while the order B-EO-S-T80 decreases over time as its score after 180 days becomes average (score = 3) and that of S-B-EO-T80 decreases rapidly over time as after 180 days it becomes unstable (score = 2) (Figure 1).

Stability as a function of storage time

Figure 2 shows that M1 formulation has a very high stability score (score = 5) after 180 days compared to M2 formulation which has a medium stability score (score = 4) and M3 formulation which has a very low stability score (score = 2).

Stability assessment as a function of pH

Figure 3 shows that M1, M2 and M3 formulations have a pH that remains stable over time. M3 formulation has a maximum pH of 10 which is higher than that of M2 formulation whose maximum pH is 8 and the latter is higher than the pH of M1 formulation which is 7.15.

Stability as a function of temperature

M1 and M2 formulations have the best stability scores (Figure 4). M1 formulation is very stable at 4˚C, 25˚C, 37˚C, and 40˚C while M2 formulation is very stable at 4˚C, 25˚C and 37˚C. At 40˚C, M2 is moderately stable and M3 is very unstable. However, M3 formulation is moderately stable at 4˚C, 25˚C and 37˚C.

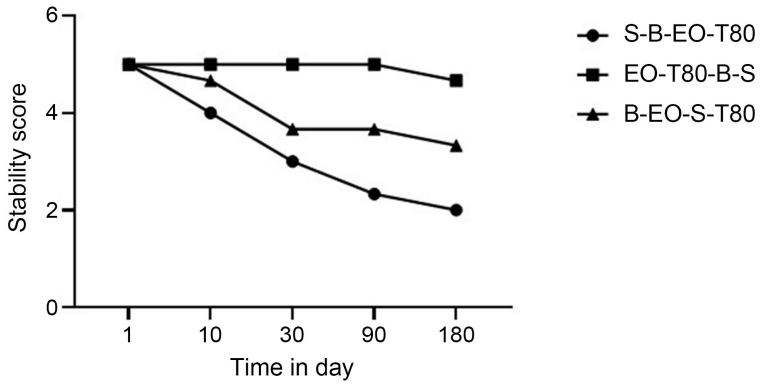

Figure 1. Stability of the formulations as a function of order of incorporation of the inputs (S: slurry, B: bicarbonate, EO: essential oil; T80: Tween 80).

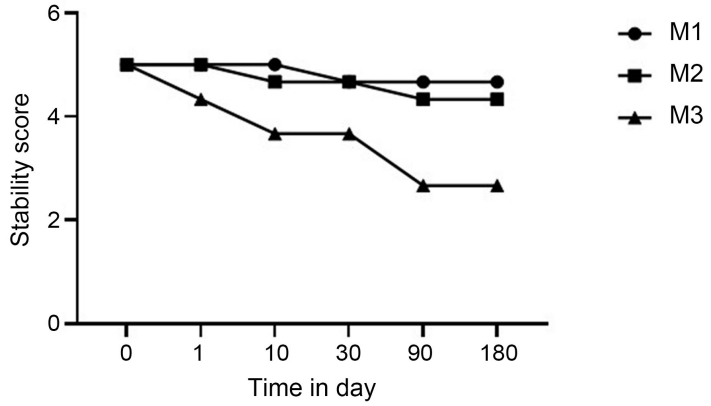

Figure 2. Stability of the different formulations as a function of time. M1: formulation 1, M2: formulation 2 & M3: formulation 3.

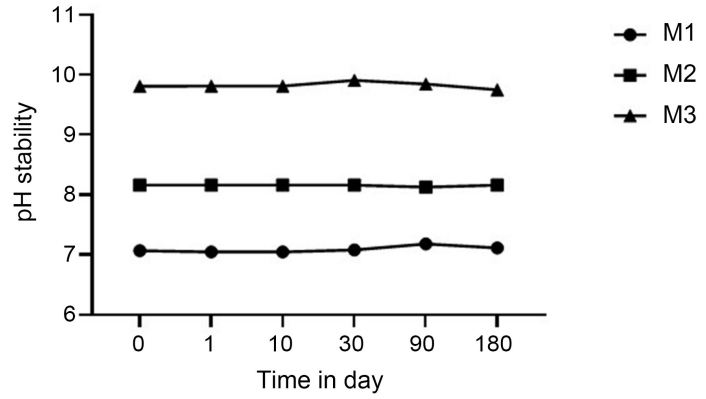

Figure 3. pH variation of the formulations as a function of Time (M1: formulation 1, M2: formulation 2 & M3: formulation 3).

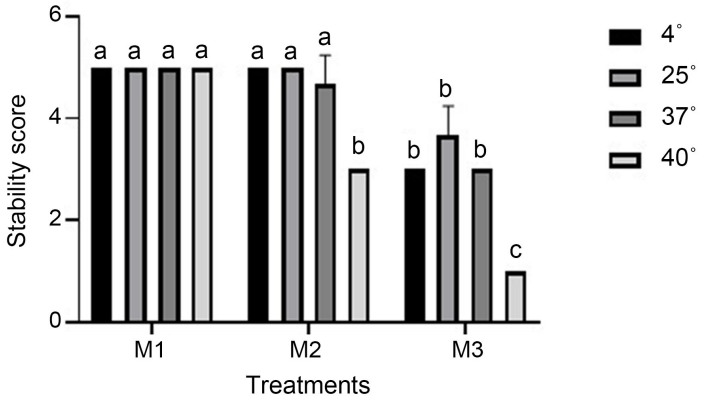

Figure 4. Stability of the different formulations as a function of temperature (M1: formulation 1, M2: formulation 2, M3: formulation 3). *The average stability score assigned to the different letters are significantly different at the probability threshold* $P < 0.05$.

3.2. Characteristics of the Different Formulations

The M1, M2 and M3 formulations have a liquid appearance, a brown color and a light texture with a lemony smell as shown in **Table 2**.

3.3. *In Vitro* Evaluation of the Fungicidal Effect of M1 and M2 Formulations on the Growth of *P. myriotylum*

The results depicted in **Figure 5** indicate a percentage inhibition of *P. myriotylum* of more than 80% in the treatments M1, M2, chemical fungicide (FC) and essential oil (EO) at the concentration 0.1% compared to sodium bicarbonate (SB). Moreover, this percentage of inhibition is dose-dependent (the higher the concentration, the greater the percentage of inhibition).

3.4. *In Vitro* Evaluation of Sporal Inhibition of *P. myriotylum* as a Function of Treatments and Concentration

It is obvious from data in **Figure 6** that the M1 and M2 formulations are as effective as the chemical fungicide with more than 80% inhibition of *P. myriotylum*

lum spore growth at the 10%, 5%, 3.33%, 1% and 0.1% concentrations. On the other hand, the formulations M1, M2 and the chemical fungicide have a lesser effect on spore growth at the 0.01% concentration with an inhibition of less than 60%.

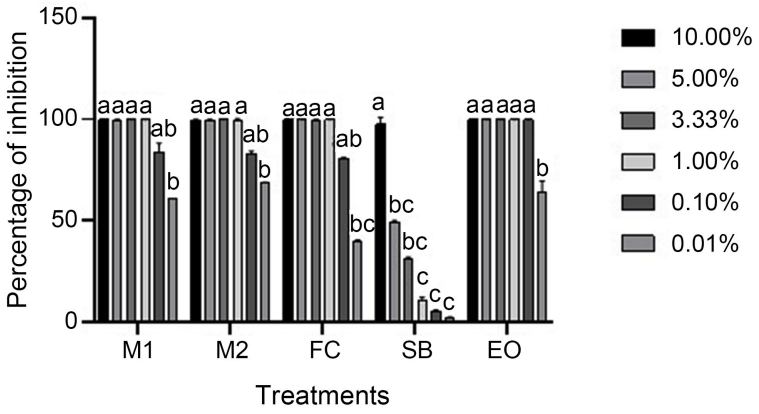

Figure 5. Percentage of inhibition of *Pythium myriotylum* as a function of the concentrations of the different treatments (M1 and M2: formulations; FC: chemical fungicide; SB: sodium bicarbonate; EO: essential oil). *The average inhibition percentage assign < ed to the different letters are significantly different at the probability threshold* $P < 0.05$.

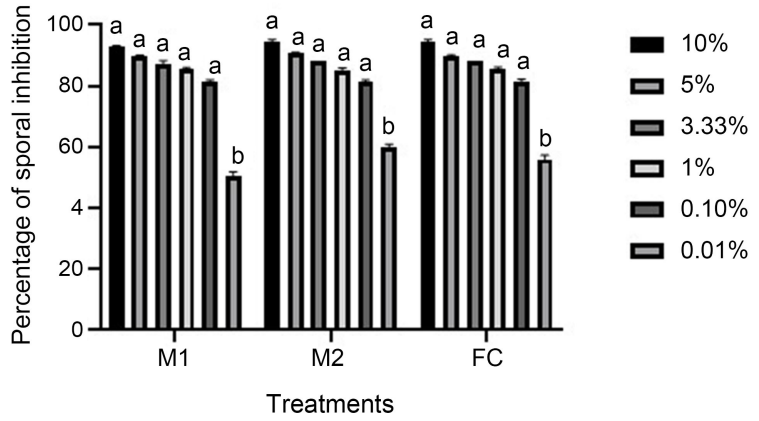

Figure 6. Percentage sporal inhibition of *Pythium myriotylum* as a function of the concentrations of the different treatments (M1: formulation 1, M2: formulation 2, FC: chemical fungicide). *The average percentage of sporal inhibition assigned to the different letters is significantly different at the probability threshold* $P < 0.05$.

Table 2. Physical characteristics of the formulations (M1: formulation 1, M2: formulation 2 & M3: formulation 3).

Characteristics	Formulations		
	M1	M2	M3
Colour	Brown	Light brown	Light brown
Odour	Lemony	Lemony	Lemony
Aspect	Liquid	Liquid	Liquid
Texture	Light	Light	Dense

3.5. Fungicidal *in Vitro* Effect of M1 and M2 Formulations on the Growth of Other Fungi

The formulations M1 and M2 at 1%, 0.1% and 0.01% concentrations respectively illustrated in Figure 7 revealed a fungicidal effect against *P. myriotylum*, *P. colocaceae*, *P. infestans*, *P. megakarya* and *F. oxysporium*. Indeed, the M1 and M2 formulations have a minimum inhibitory concentration of 0.1% in microorganism such as: *P. colocaceae*, *P. infestans*, and *P. megakarya*. However, in *F. oxysporium* and *P. myriotylum*, the minimum inhibitory concentration of the M1 and M2 formulations was 1%.

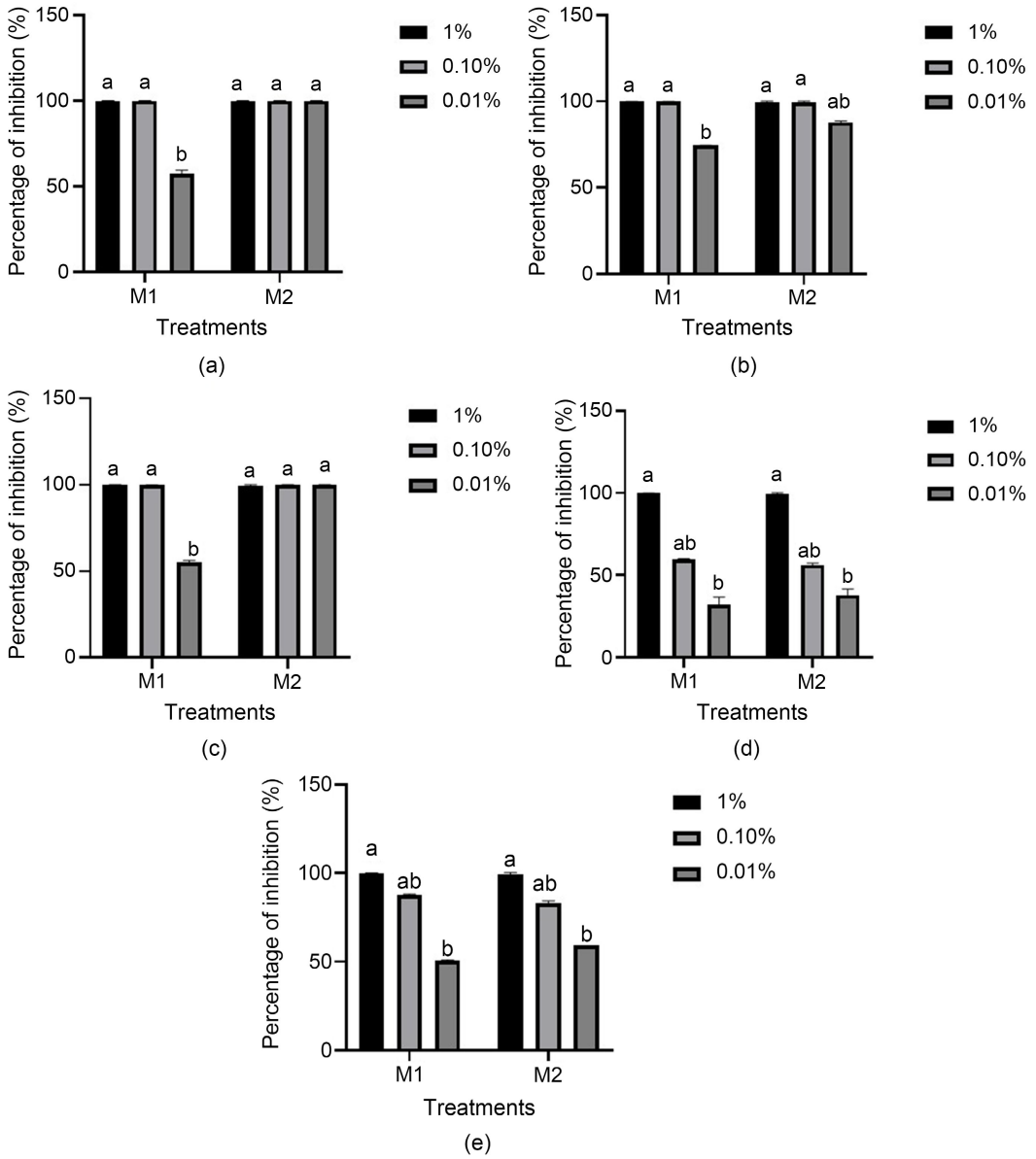

Figure 7. Percentage inhibition of Phytophthora *megakarya* (a), *Phytophtora colocacea* (b), Phytophthora *infestans* (c), *Fusarium oxysporium* (d) and *Pythium myriotylum* (e) according to treatments (M1: formulation 1, M2: formulation 2). The average percentage inhibition fungus assigned to different letters are significantly different at the probability threshold $P < 0.05$.

3.6. Mode of Action of M1 and M2 Formulations on *P. myriotylum*

✓ **Lytic activity of the formulations**

Figure 8 below shows relative absorbances of fungi at different concentrations of the formulation. The relative absorbances of the fungi in the presence of the formulation decrease over time compared to the control, which increases over time. After six hours, the 2 MIC formulation continues to decrease and the MIC formulation becomes constant while the control continues to increase.

✓ **Effect of formulations on fungal protein synthesis**

The result of Figure 9 shows that the formulation significantly ($P \leq 0.05$) inhibited the synthesis of fungal proteins compared to the negative control. This inhibition was dose dependent (the higher the concentration the greater the inhibition).

✓ **Effect of formulations on proton pump inhibition**

Figure 10 shows the inhibitory capacity of the formulations on ATPase-H+ pumps of *P. myriotylum* via the variation of pH versus time. Concerning the proton pumps, when the fungi are in the presence of the formulation, a large increase in pH values is observed during the first 15 minutes, beyond this, a slight increase in pH values is observed over time. This increase in pH reflects the inhibition of *P. myriotylum* ATP-H+ pumps by the formulation. Furthermore, the inhibition of the ATPase H+ pumps vary according to the concentrations, as the 2MIC concentration has a higher increase in pH over time than the MIC and 1/2MIC concentrations.

3.7. Effect of Treatments on the Growth of *Xanthosoma sagittifolium*

Regarding the agromorphological parameters of *X. sagittifolium* cultivars according to the treatments illustrated in Table 3, it appears that M1 & M2 formulations stimulate the growth of Xanthosoma growth compared to the chemical fungicide and the negative control. Furthermore, the M1 & M2 formulations acted in a cultivar-dependent manner. Indeed, the M1 formulation favours the growth of the red cultivar by 60.25% and white cultivar by 30.33% compared to untreated while the M2 formulation favours the growth of the white cultivar by 39.98% and the red cultivar by 33.97% compared to the non-treated plants.

3.8. Effect of Treatments on the Incidence of Roots Disease

The formulations M1 and M2 reduced the incidence of the disease on the two cultivars of *X. sagittifolium* compared to the chemically treated and the negative control (Figure 11). This decrease in disease incidence is more pronounced in the red cultivar compared to the white cultivar. Indeed, the disease incidence of the treated plants in the red cultivar is 0.045, 0.045, 0.25 and 0.85 while in the white cultivar it is 0.45, 0.47, 0.83 and 0.85 for M1, M2, FC and TN respectively.

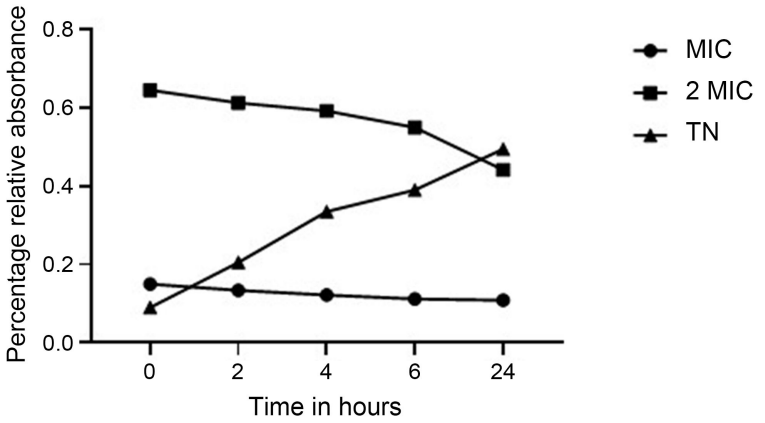

Figure 8. Relative absorbances of fungi at different concentrations of the formulation. MIC: minimum inhibitory concentration; TN: negative control.

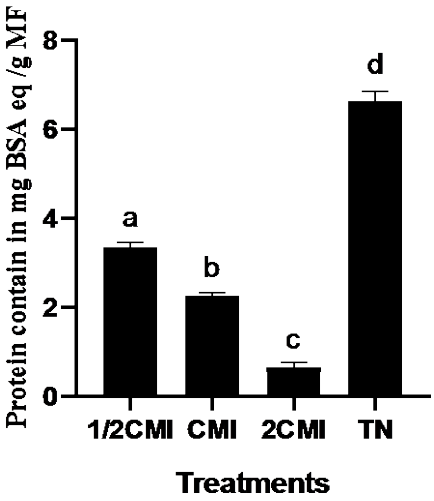

Figure 9. Fungal protein concentration in the presence of the different formulation concentrations. MIC: Minimum Inhibitory Concentration; TN: negative control. *The average total protein contents assigned to the different letters are significantly different at the probability threshold* $P < 0.05$.

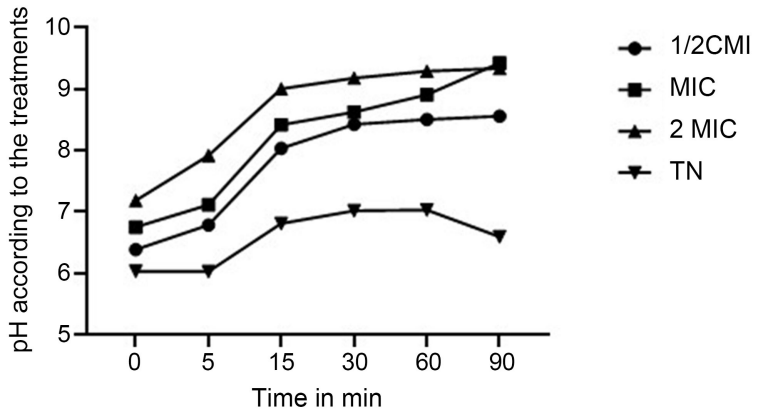

Figure 10. Effect of treatments on ATPase-H+ pumps of *P. myriotylum* as a function of time. MIC: minimum inhibitory concentration; TN: negative control.

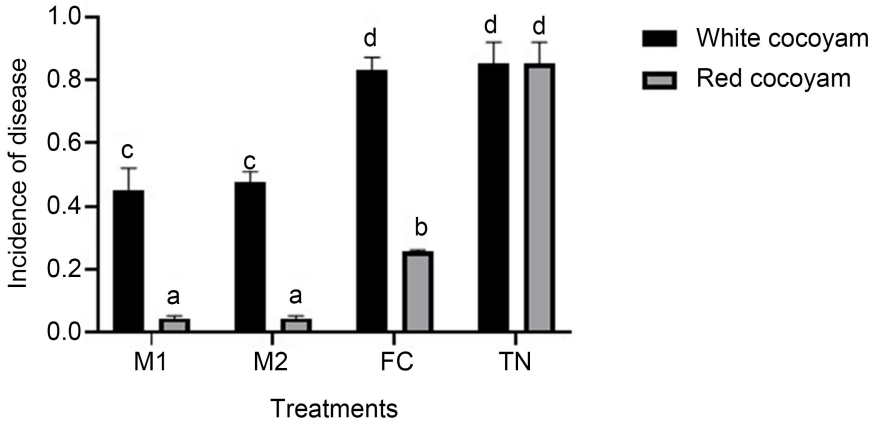

Figure 11. Incidence of disease caused by *P. myriotylum* on the white and red cultivar of *X. sagittifolium* as a function of treatments. The average incidence root rot disease assigned to different letters are significantly different at the probability threshold $P < 0.05$.

Table 3. Agromorphological parameters of white and red cultivars of *Xanthosoma sagittifolium* according to the treatments obtained by minisset.

Cocoyam treatments	Number of main roots	Length of main roots (cm)	Diameter of collar (cm)	Number of tubers	Tuber weight (g)
M1white	18.66 ± 4.51b	52.66 ± 12.66bc	2.03 ± 0.1ab	1.4 ± 0ab	0.18 ± 0.12b
M1red	29.33 ± 2.08ab	58.33 ± 15.18d	2.56 ± 0.21b	2 ± 1b	0.33 ± 0.20c
M2white	21.66 ± 10.01ab	63 ± 4.58d	2.45 ± 0.26b	1.86 ± 0.58b	0.25 ± 0.03c
M2red	17.66 ± 2.52b	48.16 ± 10.61abc	2.20 ± 0.25ab	1 ± 0a	0.1 ± 0.003ab
FCwhite	15 ± 2a	35 ± 2a	1.96 ± 0.40ab	1 ± 0a	0.3 ± 0.16c
FCred	16 ± 3a	29.5 ± 6.73ab	1.9 ± 0.1ab	1 ± 0a	0.014 ± 0.008a
TNwhite	13 ± 2.65a	34.5 ± 4.93ab	1.93 ± 0.06ab	1 ± 0a	0.076 ± 0.03ab
TNred	11.66 ± 1.53a	32.83 ± 8.005ab	1.46 ± 0.31a	1 ± 0a	0.025 ± 0.014a

4. Discussion

In this study, the objective was to prepare stable fungicide formulations, detect its antimicrobial activity, its mode of action and their protective effect on cocoyam seedlings. To achieve this, we extracted inputs such as citronella essential oil and sunflowers slurry. Once the inputs were obtained, we prepared three fungicide formulations (M1, M2 and M3), which differed in concentration of sodium bicarbonate added. The results showed that the stability varied firstly according to the order of incorporation of the inputs as the correct order that favored stability was the order EO-T80-B-S. This corroborates with the studies of [24] who showed that the order of administration of the ingredients affects the homogeneity including the stability of a mixture. Similarly, the stability of the formulations varied according to the amount of sodium bicarbonate; indeed, the amount of bicarbonate influences the size of the micelles, as the larger the micelle size, the less stable it is, which justifies the fact that the M3 formulation (15% bicarbonate) is less stable than M1 (5% bicarbonate) and M2 (10% bicar-

bonate). Furthermore, the amount of sodium bicarbonate influences the pH of these formulations, as the M1 and M2 formulations with a pH of 7 and 8 respectively were the most stable compared to the M3 formulation with a pH of 10. This result confirms that of [25] who showed that the stability of the formulations decreases as the pH increases.

Furthermore, temperature is one of the most important factors in the stability of fungicides. A 10˚C increase in storage temperature can lead to a 2-to-5-fold increase in the rate of degradation reactions. Our results showed that the M1 and M2 formulations were most stable at 4˚, 25˚, 37˚C and 40˚C temperatures compared to the M3 formulation because the M1 and M2 formulations had a high stability score. This result correlates with that of [26] who showed that temperature influences the chemical or physical stability of the active ingredient of a product.

From the results obtained in the *In vitro* tests, the stable treatments M1 and M2 significantly influenced the mycelial growth of the *P. myriotylum* in the same way as chemical fungicide (FC). The efficacy of the fungicide's formulations (M1 and M2) on the development of this microorganism varied according to the concentrations, as the latter (M1 and M2) have a minimum inhibitory concentration of 1% on the development of *P. myriotylum*, but have a reduced fungistatic effect of 0.01% on *P. myriotylum*. This corroborates with the studies of [27] on NECO (biofungicide whose active ingredient in *Ocimum gratissum*) whose effectiveness at the concentration of 100ppm was reduced to 65% on pestalotia and heterocornis pathogens.

Formulations M1 and M2 showed broad-spectrum antifungal properties, and the potential of the latter to control pathogens. The growth of all fungal pathogens studied was significantly inhibited ($P < 0.05$) at increasing concentrations of the M1 and M2 formulations. The response of different fungal pathogens to the formulations, however, varied with the concentrations of the formulations. *P. colocaceae* pathogen was the most sensitive with the highest growth reduction at the lowest concentration tested (0.01%), while F. *oxysporum* pathogen was the least sensitive. Nevertheless, the growth of all fungal pathogens was totally inhibited at 1% of the formulations. These results correlate with those of [28] [29] which showed that citronella essential oil and *Prosopis Africana* leaf extract have a fungitoxic effect on the mycelial growth of *F. oxysporium* (banana wilt), *C. gloeosporides* (mango anthracnose), *C. falcatum* (sugarcane rot) and *Neopestalotiopsis spp*. (mango leaf spot) evaluated *In vitro* trials. Moreover, these results correlate with those of [30] who showed that the essential oil of *C. citratus* at 1% totally prevented the development of *B. oryzae* and any mycelial production. The inhibitory effect of these fungicidal formulations on the microorganism used, particularly on *P. myriotylum* could also be due to the presence of sodium bicarbonate. The latter has the ability to reduce the turgor pressure of the fungal cells resulting in collapse and shrinkage of hyphae and spores, and consequently the inability of the fungus to sporulate. This result was consistent with the observation of [31], who found that sodium bicarbonate was very effective in con-

trolling sweet cherry rot.

Secondary metabolites, which have pharmacological properties, use different modes to exert their actions on fungi. Thus, they can act at the level of the cell wall, at the level of membrane constituents, or can penetrate the cytoplasm where they could act at the level of organelles and nucleic acids or interfere with biochemical processes important for the life of the microorganism [21]. The mode of action of M1 and M2 formulations on the fungus *P. myriotylum* was evaluated and the effect of these formulations on the integrity of the fungal membrane was determined. Measurement of fungal absorbance kinetics showed a reduction in the absorbance of *P. myriotylum* in the presence of the M1 and M2 formulations and this decrease in the absorbance of the fungal suspension is generally a characteristic of fungal lysis [21]. The percentage absorbance decreased in a dose-dependent manner with time, which would indicate a good fungilytic activity of the formulations.

Similarly, all concentrations of the fungicide formulations (2 MIC, MIC and 1/2 MIC) significantly inhibited protein synthesis in *P. myriotylum* compared to the control. The inhibitory power of the formulations on fungal protein synthesis could be explained by the richness of these formulations in citronella essential oil, which is known for its ability to inhibit mycelial growth of fungi [32]. In addition, the increase in pH of the fungus treated with the formulation compared to the control would reflect the efficiency of the fungal proton pumps to regulate the intracellular pH, which could be explained by the presence of sodium bicarbonate, which causes an increase in pH and osmotic pressure in the cells, both of which lead to detrimental conditions for fungal spores [33]. This inhibitory effect of the pumps by the fungicide formulations suggests that the pumps are one of the potential targets of action of the formulation.

The efficacy of these formulations was tested on two cultivars of *X. sagittifolium* commonly used in Cameroon, white (susceptible) and red (tolerant) *X. sagittifolium* cultivars. The two formulations enhanced significantly the growth of cocoyam seedlings of the two cultivars of *X. sagittifolium* under greenhouse conditions and greatly reduced the severity of *P. myriotylum*. The ability of the treatments (M1 and M2) to reduce the incidence of the disease could be explained by the antifungal properties of these different compounds, which would allow them to stop or slow down the mycelial production of the pathogenic fungus. The presence in the formulations of *Tithonia diversifolia* extract which has a systemic action and which according to the studies of [34] and [35], rich in phenols, tannins and flavanoids, has fungicidal importance. This extract would be responsible for inhibiting of *P. myriotylum* by binding to their protein molecules, acting as chelating agents, altering their biochemical systems and preventing the use of nutrients available to the organisms.

5. Conclusion

Our study allowed us to design tropical plant-based formulations, study their stability, measure their antifungal efficacy, determine their mode of action and

show their effect on Xanthosoma growth. The stability of these formulations depended on the order of incorporation of the inputs and the correct order was EO-T80-B-S. Formulations M1 and M2 were the most stable because they had a high stability score at 40˚C, 37˚C, 25˚C and 4˚C temperatures, their pH varied between 7 and 8 and they had a fine micelle size. The M1 and M2 formulations have a minimum inhibitory concentration between 0.1% and 1% *on P. megakarya*, *P. infestans*, *P. colocaceae*, *F. oxysporium* and *P. myriotylum*. They act on the fungus by lysing the membrane, by inhibiting fungal synthesis and by inhibiting proton pumps. M1 and M2 formulations have better efficiency and efficacy in inhibiting *P. myriotylum* at both *in vitro* and *in vivo* levels. It would therefore be interesting to verify the effect of these formulations on promoting the growth of Xanthosoma and the biochemical and molecular defense systems of Xanthosoma after infection by *P. myriotylum* in order to demonstrate the stimulatory effect of these formulations

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Conflicts of Interest

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

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