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In vitro antifungal activities of fungicides based of plants essentials oils (NECO, ASTOUN and FERCA) and phosphorous acid on *Phytophthora katsurae* (Pythiaceae), causal agent of the premature nut fall and the heart rot of the coconut tree, in Côte d'Ivoire

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ABSTRACT

The present study aimed at evaluating the antifungal activities of NECO, ASTOUN and FERCA fungicides based of plants essentials oils against *Phytophthora katsurae* a major constraint in coconut plantations in Côte d'Ivoire. *In vitro*, the inhibition properties of these fungicides was evaluated at different concentrations 50, 100, 250, 500, 1000, 1500, 2000 and 2500 ppm and compared to a synthetic fungicide (phosphorous acid) at 1, 5, 10, 25, 50, 100 and 150 ppm on the radial growth of the mycelium of an isolated pathogen from a diseased nut. The mycelial growth was measured every day during 10 days in the Petri dishes. The results showed inhibition rates ranging from 1.48% to 100%. The biopesticides NECO and ASTOUN were effective against *Phytophthora katsurae* at respectively 149.14 and 272.38 ppm compared to phosphorous acid. NECO was fungitoxic at 2500 ppm and fungistatic at 1500 and 2000 ppm. Phosphorous acid was simply fungicidal at 150 ppm. Based on these results, these fungicides based of plants essentials oils could be used as biological control of *Pnhytophthora katsurae* in coconut plantations in Côte d'Ivoire.

Keywords: *Phytophthora katsurae*, coconut plantations, fungicides based of essentials oils, biological control, Côte d'Ivoire.

INTRODUCTION

Species like *Phytophthora*, are Oomycete pathogens, which cause significant damage to plants throughout the world (Wong, 2006). They have traditionaly been placed under family Pythiaceae in the Kingdom Chromista, but now current knowledge suggest them closely related to heterokont algae attached to the monophyletic Kingdom Stramenopila (Restrepo et al., 2016).

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In Côte d'Ivoire, *Phytophthora* species encountered are mainly in cocoa and walnuts production (Allou and de Franqueville, 2001; Coulibaly et al., 2013). Thus, in cocoa production, *Phytophthora palmivora* and *Phytophthora magakarya* cause field losses respectively from 10 to 15% and 40 to 60% (Pokou et al., 2008). In walnuts production, *Phytophthora katsurae* causes the tree's premature nuts fall and the meristem dieback called heart rot. These diseases quickly lead to the death of the tree and to crop losses up 1 to 35% on station (Allou et al., 2003). To fight against this parasitic constraint, several means of control have been recommended, particularly prophylactic, genetic and chemicl control with synthetic products (Allou and de Franqueville, 2001; Mpika et al., 2009b; N'Goran et al., 2010; N'Goran et al., 2020). However, these different methods have many limitations. Thus, chemical control with synthetic products has some disadvantages, particularly the high cost of treatments, the resistance of pathogens to certain molecules (Akantetou et al., 2020).

Facing this situation, the search for other means of control (Ahouansou et al., 2019) that are both effective and respectful to the standards on the local Ivorian market is necessary. To this end, several research works using natural substances such as extracts from local plants have made it possible to fight effectively against several types of plant pests (Kassi et al., 2014 ; Yala et al., 2016; Camara et al., 2017 ; Fofana et al., 2020; Nyaka et al., 2021). This has led to the formulation of many biopesticides (Camara et al., 2007; Camara et al., 2010; Yala et al., 2017; Akantetou et al., 2020).

This study aimed at proposing a means of biological control through the use of plant extracts against *Phytophthora katsurae* in order to maintain the production of coconut plantations.

MATERIALS AND METHODS

Materials

The fungal material used was an isolate of *Phytophthora katsurae* isolated from a diseased nut collected at the Marc Delorme coconut station of the National Agronomic Research Center (CNRA) located at Port-Bouët in the Autonomous District of Abidjan, in Ivory Coast. The biofungicides were formulations based on essential oils from hydrodistillation of leaves and coded NECO, ASTOUN and FERCA. As for the synthetic fungicide, it consisted of phosphorous acid used as a positive control.

Methods

Culture and conservation of Phytophthora katsurae strains

Both modified V8 and Ribeiro media were used. Modified V8 medium was used for refreshing and preservation of *Phytophthora katsurae* strain. Modified Ribeiro medium was used for *in vitro* evaluation of the antifungal potential of biopesticides and phosphorous acid. One liter of modified V8 medium is composed of 45 ml of V8 vegetable juice, 21 g of Agar-agar and 955 ml of distilled water (N'goran, 2010). After autoclave sterilization at 1.5 bars at 121 °C for 30 min, the resulting solution after slight cooling was dispensed into 90 mm diameter Petri dishes under a laminar flow hood in the presence of flame. Using a cookie cutter, 6 mm diameter mycelial discs were taken from Petri dishes containing *Phytophthora katsurae* cultures and transplanted into the center of Petri dishes containing V8-agar medium. As for the modified Ribeiro medium, it has a composition containing 4.5 g of glucose, 0.1 g of L-asparagine, 0.15 g of potassium nitrate (KNO_3), 1.36 g of potassium dihydrogen phosphate (KH_2PO_4), 0.5 g of Magnesium sulfate ($MgSO_4$, $7H_2O$) and calcium chloride ($CaCl_2$) as base solution A, a Microelement solution of 41.1 mg Sodium Molybdate (Na_2MoO_4, H_2O), 87.8 mg Zinc Sulfate ($ZnSO_4$, $7H_2O$), 7.85 mg Copper Sulfate ($CuSO_4$, H_2O), 15.4 mg Manganese Sulfate II ($MnSO_4$, H_2O), 0.5 mg Sodium Tetraborate ($Na_2\ B_4O_7$), 1000 ml distilled water as solution B and then a ferric solution of 50 mg Iron III chloride, 2.6 mg EDTA, 1.5 mg KOH as solution C to which 17 g Agar-Agar is added to obtain the solid medium. The pH of the medium was adjusted to 6.2 with 6 M KOH using a pH meter before autoclaving at 1.5 bar for 30 min. After autoclaving, 1 ml of sterilized

Thiamine solution was added to the medium while cooling to approximately 50 °C before being dispensed into the Petri dishes (King, 2007).

Preparation of the test media

The 100 ml Ribeiro media were prepared by autoclaving at a temperature of 121 °C under a pressure of 1 bar for 30 min. After cooling the media at room temperature to the supercooling temperature, the fungicides were added to the media to obtain the concentrations of 1; 5; 10; 25; 50; 100 and 150 ppm for the phosphorous acid fungicide and 50; 100; 250; 500; 1000, 1500; 2000 and 2500 ppm for the NECO, ASTOUN and FERCA biological fungicides. Ribeiro media amended with the different fungicides were homogenized and distributed in a laminar flow hood in 90 mm diameter Petri dishes at a rate of 18 ml per dish. The controls were dishes containing no fungicide. For each fungicide, five Petri dishes were used per concentration.

Seeding and incubation

Using a cookie cutter, 6 mm diameter mycelial discs were taken from 7 day old cultures and seeded in the center of fungicide-treated and non-fungicide-treated Petri dishes. The cultures were sealed with the stretch film and incubated at 25°C for 10 days.

Assessment of mycelial growth

Radial growth was measured every day in millimeter, excluding the plug until 10 days. Measurements of mycelial radial growth were made along two perpendicular lines drawn on the reverse side of each Petri dish that intersected at a point in the middle of the mycelial ring. The effect of fungicides on mycelial growth of the fungus was determined through the rate of mycelial growth inhibition calculated by the formula of Hmouni et al. (1996):

$$I(\%) = \frac{Do - Dc}{Do}$$

(I = inhibition rate; Do = average diameter of mycelial growth of colonies in control boxes; Dc = average diameter of mycelial growth of colonies at concentration (c) of synthetic fungicide or biological fungicides).

From the ed50v10 (1) software, the mycelial growth inhibition rates are transformed into probit values with the regression lines in the form of y = a log x + b according to the transformed formula of Paranagama et al. (2003) (Y the probit, a the regression coefficient, b the constant, x the fungicide concentration, log the decimal logarithm). The equations of these regression lines allowed us to determine the IC_{50} and IC_{90}, which are the fungicide concentrations that reduce the mycelial growth of the fungus by half 50% and by 90% (Oxenham et al., 2005; Neri et al., 2006).

Test of fungitoxicity of fungicides

When no mycelial growth has been observed for a given concentration of fungicide, then the mycelial disc is transplanted into a new Petri dish containing Ribeiro culture medium without addition of fungicide. All these plates are kept under the same conditions. If there is no mycelial regrowth the concentration is said to be fungicidal and if not it is said to be fungistatic (Soro et al., 2010).

Statistical analysis

All data obtained were entered into Excel, calculated and analysed with Statistica 7.1. The calculation of the inhibition rates allowed to evaluate the occupation of the mycelial growth of the strains on the culture media. An Analysis of Variance with a classification criterion was used to study the sensitivity of the *Phytophthora katsurae* strain to the different fungicides used. The Newman-Keuls test with a 5% probability allowed to classify the proportions obtained.

RESULTS

Effect of different concentrations of the synthetic fungicide phosphorous acid on the mycelial growth of *Phytophthora katsurae*

On day 1, a 100% inhibition rate was observed with the 50, 100 and 150 ppm concentrations. For 150 ppm, this inhibition rate remained constant until day 10. For the 100 ppm concentrations, a slight decrease in the inhibition rate was observed from the 9th day up to 98.22% on the 10th day, on the other hand with the 50 ppm concentration from the 3rd day a progressive decrease in the inhibition rate was observed reaching 76.72% on the 10th day. With the concentrations 1; 5; 10 and 25 ppm, on the first day the inhibition rates are lower than 80 %. At the concentration of 1 ppm the

phosphorous acid had no effect on the mycelial growth, from the first day, a practically null inhibition rate until the 10th day was observed. For concentrations of 5, 10 and 25 ppm the inhibition rates decreased progressively with time. The inhibition rates recorded at day 10 for these are respectively 5.48%; 1.48%; and 10% (Figure 1).

Effect of different concentrations of the biological NECO fungicide on the mycelial growth of *Phytophthora katsurae*

For NECO biological fungicide, concentrations 1500, 2000 and 2500 ppm showed complete inhibition of mycelial growth from day 1 to day 10. For the concentrations 50; 100; 250; 500 and 1000 ppm the inhibition rates progressively decreased with time. For the latter, on the last day, the inhibition rates were respectively 36.39%; 39.57%; 52.67%; 53.33% and 62.86% (Figure 2).

Effect of different concentrations of the biological ASTOUN fungicide on the mycelial growth of *Phytophthora katsurae*

For ASTOUN biological fungicide, the daily mycelial growths of *Phytophthora katsurae* observed decreased progressively with time. However, at 2500 ppm this rate was 89.11% on the last day and was higher compared to the concentrations 50; 100; 250; 500; 1000; 1500 and 2000 ppm whose inhibition rates were respectively 5.74%; 35.24%; 39.06%; 43.81%; 54.29%; 61.54% and 65.22% (Figure 3).

Effect of different concentrations of the biological FERCA fungicide on the mycelial growth of *Phytophthora katsurae*

With FERCA biological fungicide, all concentrations resulted in a progressive decrease of the inhibition rate. Thus, the highest inhibition rates were recorded with the concentrations of 2000 and 2500 ppm, with 54.07% and 80.41% respectively. For the concentrations of 50; 100; 250; 500; 1000 and 1500 ppm, the inhibition rates recorded were 7.17; 8.87; 14.12; 16.79; 22.49 and 15% (Figure 4).

Comparative effects of natural fungicides and synthetic fungicide on *in vitro* mycelial growth of *Phytophthora katsurae*

The different fungicides used acted differently on the mycelial growth of *Phytophthora katsurae*. A highly significant difference (F=1095.677; P=0.000) was observed between the fungicides for all concentrations used. Thus, NECO biological fungicide recorded the best inhibition rate with a value of 74.56% followed by ASTOUN and phosphorous acid fungicides with respective inhibition rates of 66.80% and 56.06%. The biological fungicide FERCA with an inhibition rate of about 48.55% was the one that gave the lowest inhibition rate (Figure 5).

50 and 90% inhibitory concentrations (IC_{50} and IC_{90}) of mycelial growth of *Phytophthora katsurae*

The 50 and 90% inhibitory concentrations of mycelial growth of *Phytophthora katsurae* differed according to the fungicides. Among the organic fungicides, NECO recorded the lowest concentration (149.14 ppm) that inhibits 50% of the mycelial growth of the pathogen. This concentration is very low compared to the IC_{50} of the synthetic fungicide which is 810.32 ppm. The highest IC_{50} of 1016.39 ppm was obtained with FERCA. The IC_{90} was 1662.9; 1793.12 and 2269.28 ppm respectively for, NECO, ASTOUN and FERCA (Table 1).

Test of fungitoxicity of fungicides

Among the biological fungicides no mycelial growth was observed for the concentrations of 1500; 2000 and 2500 ppm of NECO. For phosphorous acid at the concentration of 150 ppm there was no mycelial growth. When the explants from these concentrations were transplanted on Ribeiro medium without fungicide, there was no mycelial growth for the explant from the 2500 ppm NECO concentration, contrary to the 150 ppm phosphorous acid concentration where the fungus resumed its growth. The NECO fungicide is therefore fungitoxic at 2500 ppm and fungistatic at 1500 and 2000 ppm, whereas the phosphorous acid is simply fungicidal at 150 ppm (Table 2).

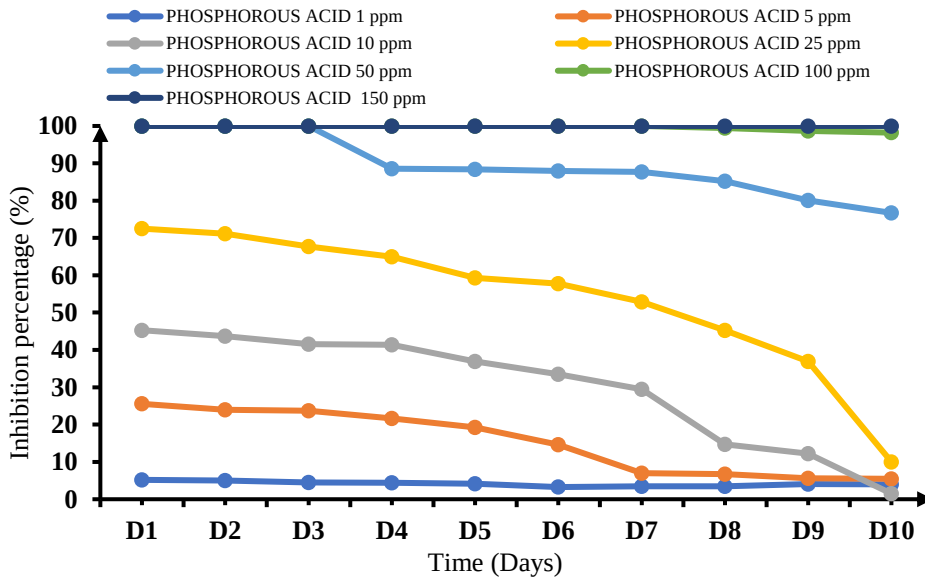


Figure 1: Variation in the daily mycelial growth inhibition rate of *Phytophthora katsurae* according to different phosphorous acid concentrations.

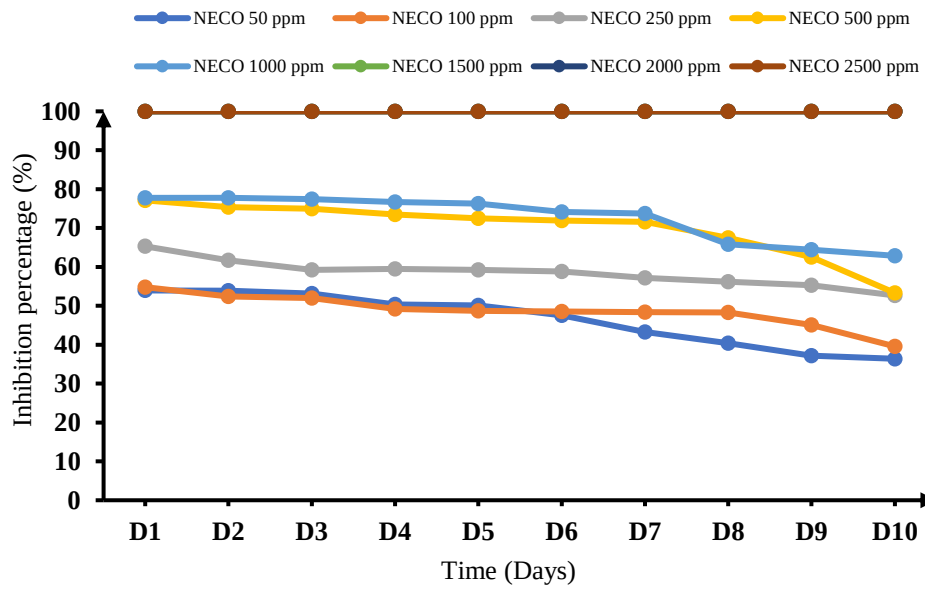


Figure 2: Variation in the daily mycelial growth inhibition rate of *Phytophthora katsurae* according to different concentrations of NECO biological fungicide.

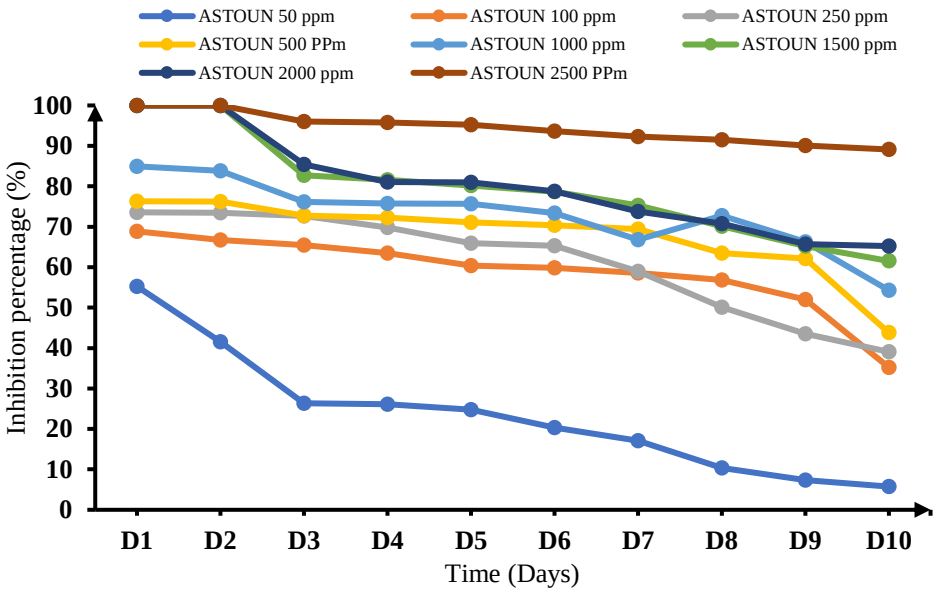


Figure 3: Variation in the daily mycelial growth inhibition rate of *Phytophthora katsurae* according to different concentrations of ASTOUN biological fungicide.

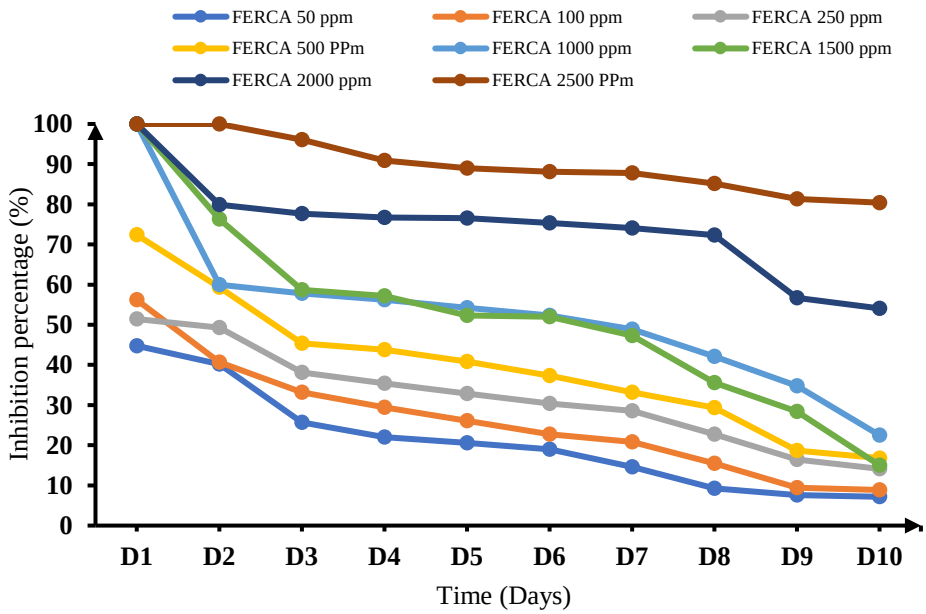
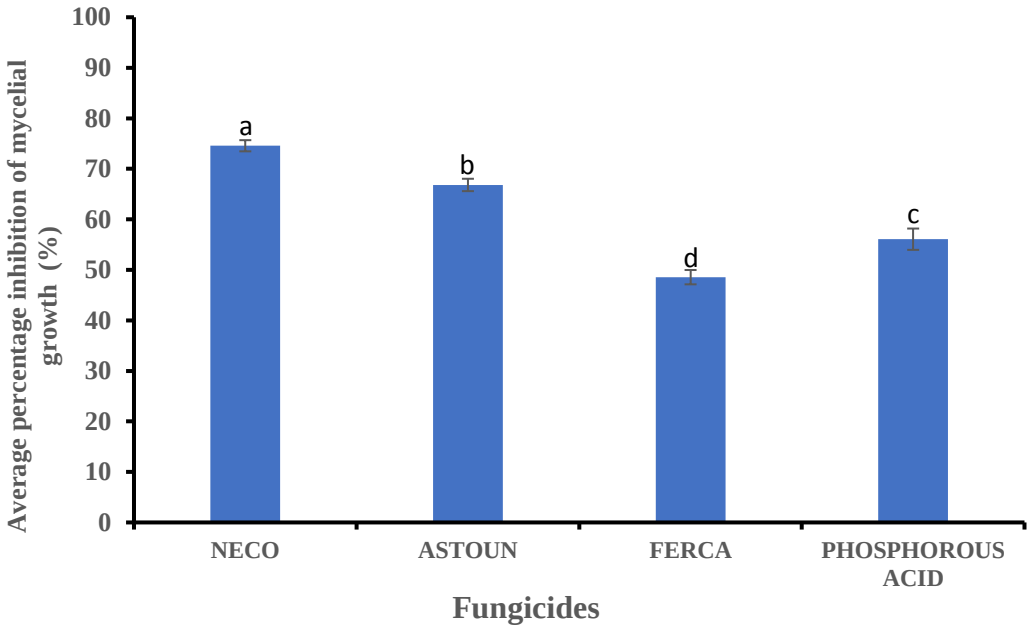


Figure 4: Variations in the daily mycelial growth inhibition rate of *Phytophthora katsurae* according to different concentrations of FERCA biological fungicide.



Histograms bearing the same letters are not significantly different at the 5% level.

Figure 5: Average inhibition rate of mycelial growth of *Phytophthora katsurae* according to the biological fungicides NECO, ASTOUN, FERCA and the synthetic fungicide Phosphorous acid.

Table 1: IC_{50} and IC_{90} inhibitory concentrations of NECO, ASTOUN, FERCA biological fungicides and the synthetic fungicide Phosphorous acid.

FUNGICIDES	IC_{50} (ppm)	IC_{90} (ppm)
NECO	149.14	1662.9
ASTOUN	272.38	1793.12
FERCA	1016.38	2269.3
PHOSPHOROUS ACID	810.316	1535.6

Table 2: Fungitoxicity of fungicides.

FUNGICIDES	Concentration (ppm)	Mycelial growth
NECO	1500	+
NECO	2000	+
NECO	2500	-
PHOSPHOROUS ACID	150	+

(+): Resumption of growth; (-): No resumption of growth.

DISCUSSION

In this study, the effects of the biopesticides NECO, ASTOUN and FERCA were evaluated and compared to the synthetic fungicide, the phosphorous acid. Thus, the Phosphorous acid significantly reduced the mycelial growth of *Phytophthora katsurae* from 50 ppm. These results are similar to those obtained by N'goran et al. (2010) who showed the effectiveness of the phosphorous acid in the control of *phytophthora katsurae*.

Like phosphorous acid, the biological fungicides acted significantly on the mycelial growth of *Phytophthora katsurae*. These results are in agreement with those of Fofana et al. (2020) on the antifungal activity of these three biological fungicides on *Phytophthora palmivora* responsible for brown rot on the cocoa tree. As these different biological fungicides are essential oil based formulations, the antifungal, antibacterial and insecticidal activity of these essential oils has already been proven during the work of Camara et al. (2007) on *Mycosphaerella fijiensis* which caused leaf diseases of banana and plantain in West Africa. The effectiveness of these biological fungicides was according to the used concentrations. Indeed, NECO totally inhibited the growth of *Phytophthora katsurae* from 1500 ppm. These results are similar to those obtained by Silue et al. (2018) who showed the efficiency of NECO biological fungicide in inhibiting the mycelial growth of *Colletotrichum* at concentrations of 300, 400, 500 ppm. Also, the work of Yeo (2017) showed that NECO completely reduced the mycelial growth of *Sclerotium rolfsii* at the concentrations of 3000, 5000, 7000 and 10000 ppm. The antifungal activity of NECO would be due to its chemical composition. NECO is formulated from essential oil whose active components are thymol (Kassi et al., 2014) and eugenol. Thymol and eugenol are responsible for the fungicidal (Chami, 2005) and bactericidal activity of essential oils containing them (Cox et al., 2000). Also, *in vitro*, Camara et al. (2007), showed that NECO inhibits spore production and mycelial growth of *Deightoniella torulosa* strains. Concerning the biofungicide ASTOUN, the mycelial growth was reduced by half from 1000 ppm. These results are contrary to those of N'Goran (2017) who showed that the ASTOUN biofungicide at 100 ppm inhibited mycelial growth by half. This difference would be due to the degree of sensitivity of the fungus to different concentrations of this biological fungicide. According to Gabriel et al. (2013) and El Amri et al. (2014) the biological activities can be explained first by the chemical composition of these oils, but also, by the quantitative and qualitative variability of its components. The antifungal activity of ASTOUN would be due to its chemical composition. The active components of ASTOUN are thymol and citronellol. The difference in activity between NECO and ASTOUN biological fungicides would then be due to the absence of eugenol in the chemical composition of ASTOUN. FERCA biological fungicide was the least effective, inhibiting mycelial growth of *Phytophthora katsurae* by half only at the concentration of 2000 ppm. Thus, the biopesticides NECO and ASTOUN could be recommended as biological control of *Phytophthora katsurae* of coconut tree.

Conclusion

Mycelial growth of *Phytophthora katsurae* was dependent on the used products and concentrations. Among the biological fungicides used, NECO and ASTOUN showed an efficiency in the control of *Phytophthora katsurae* at 149.14 ppm and 272.38 ppm respectively compared to the phosphorous acid and synthetic fungicide. The use of these biopesticides could be a solution in the control of immature nut drop and heart rot of coconut tree, especially through the implementation of efficiency tests in plantations.

COMPETING INTERESTS

The authors declare that they have no competing interests in the publication of this work.

AUTHORS' CONTRIBUTIONS

KSBN defined the theme and objectives of this study. He guided the methodological approach and the results obtained. He participated in the analysis of the data and the drafting of the proposed manuscript. BC and

CAN contributed to the definition of the methodological approach and the obtained results. NK collected the field and laboratory data and participated in the writing of this manuscript. MST, DO and SA corrected the form and content of the proposed manuscript. All authors read and approved this manuscript.

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