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Int. J. Biol. Chem. Sci. 13(4): 1938-1959, August 2019

ISSN 1997-342X (Online), ISSN 1991-8631 (Print)

International Journal of Biological and Chemical Sciences

***Original Paper*** http://ajol.info/index.php/ijbcs http://indexmedicus.afro.who.int

# Inhibitory effects of aqueous and ethanolic extracts of pepper (*Capsicum annuum*) on the development of necrosis caused by *Phytophthora megakarya* in *Theobroma cacao*

Claude SIMO[1,4*], Alice Virginie TCHIAZE IFOUE[1], Liliane Corine NOUAGE TAYOUKAM[1], Romuald NSOUGA AMOUGOU [2], Victorine LOMBEKO[2], Stephanie Alvine NJONZO-NZO[1] and Pierre François DJOCGOUE[3,4]

[1]*Department of Plant Biology, Faculty of Science, University of Douala, Cameroon.*
[2]*Research Institute for Agricultural Development, Yaoundé, Cameroon.*
[3]*Department of Plant Biology, Faculty of Science, University of Yaoundé 1, Cameroon.*
[4]*Laboratory of Plant Physiology, Department of Biological Sciences, Higher Teacher's Training College, University of Yaoundé 1, Cameroon.*
[*]*Corresponding author; E-mail: simoclaude@yahoo.fr / simoclaude@univ-douala.com; Phone: (+237) 677 58 92 87/691 78 36 07*

## ABSTRACT

The cocoa tree (*Theobroma cacao* L.), also called "food of the gods", is a plant mainly cultivated for its beans. However, the cultivation of this plant is confronted with many parasitic attacks among which, attack due to *Phytophthora megakarya* which causes the most yield loss. The Control of this pathogen consisted in involving the use of aqueous and ethanolic extracts of pepper and two strains of *Phytophthora megakarya* in order to improve cocoa tolerance against the attack of this pathogen. These aqueous and ethanolic extracts were prepared in the laboratory and incorporated into Potato Dextrose Agar and Small Pea Agar culture media at concentrations (g / ml) of $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$, each containing a strain of *Phytophthora megakarya*. The results obtained revealed that all pepper treatments significantly inhibited the growth of *Phytophthora megakarya* with a 100% inhibition rate in the aqueous extract regardless of the strain used and inhibition rates of 89.33%. ; 63.35%; 54.39%; 42.86%; 100%; 70.22% and 57.54% for the ethanolic extract depending on the concentrations applied. *In vivo* treatment of leaf discs and pods detached prior to infection with *Phytophthora megakarya* resulted in significant inhibition, thereby reducing the incidence rate of disease severity. The values of this incidence vary from 0.1 to 1.2 for a percentage ranging from 0.5% to 20% of the severity of the disease depending on the nature of the extract of applied pepper. At concentrations (g / ml) $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$, the aqueous extract completely inhibited the growth of mycelium and the development of *Phytophthora megakarya* spores while the ethanolic extract of pepper showed its inhibitory power at the concentration $10^{-1}$ g/ml. The use of crude aqueous extracts or even diluted of pepper can therefore be considered in the fight against black pod desease in Cameroon.


**Keywords**: *Phytophthora megakarya*, *Theobroma cacao*, *Capsicum annuum*, percentage of inhibition, biological control, black pod desease.



DOI: https://dx.doi.org/10.4314/ijbcs.v13i4.3

## INTRODUCTION

Cocoa (*Theobroma cacao* L.) is a perennial tropical plant of the family *Malvaceae* and native to the Amazon rainforest (Pokou et al., 2019). It is mainly grown in the tropics of West and Central Africa, Southeast Asia, and Central and South America. This diploid tropical fruit species (2n = 2x = 20) is an economically important agricultural product for millions of people around the world (Anushka and Dunwell 2018; Morrissey et al., 2019). This plant is grown by about 6 million farmers around the world and the livelihood of more than 40 million people depends on cocoa (Anushka et al., 2018). The majority of world cocoa production (about 80% to 90%) comes from smallholders whose main activity is the production of the beans that are the source of raw material for the chocolate and confectionery industry (Squicciarini and Swinnen, 2016 ; Navarro et al., 2017).

In Cameroon, more than 80% of cocoa is grown by smallholders (Duguma et al., 2001). Cameroonian cocoa farming has a great phenotypic and morphological diversity. This crop is confronted with several problems, among which is attacks of bio-aggressors such as diseases and pests. Among the diseases, the black pod desease due to *Phytophthora megakarya* constitutes the major constraint to this crop (Ndoumbé, 2001), this disease causes more than 50% of losses in Central Africa , while in some cocoa plantations in Cameroon, field losses is of the order of 50-80% and can even reach 100% if no control measures are taken.

To fight against this plague, producers resort to chemical treatment by the use of fungicidal molecules proposed by phytosanitary firms. But the use of fungicides remain constraining because of its expensive prices and and the practical aspect of the work which is very difficult for the farmer (Ahoudi et al., 2018; Konate et al., 2018; Ouattara et al., 2018). To this, must be added the requirements of the quality of cocoa that must be preserved and the protection of the environment which does not encourage the unrestrained application of fungicides (Bowers et al., 2001). The assessment of cocoa resistance to *Phytophthora sp* from organs other than pods significantly reduced breeding time and increased the genetic improvement of cacao. However, selected clones or hybrids have tolerance only against parasite rather than complete resistance. The strategy used is the less restrictive integrated pest management method, which is less costly for peasants and respectful of the environment. This strategy includes good cultural practices, selection of *Phytophthora megakarya* tolerant varieties, use of antagonistic organisms and natural substances. The evaluation of the activity of the aqueous and ethanolic extracts of pepper against *Phytophthora megakarya* the causal agent of black pod desease is part of the search of natural control methods against this disease. The present work aimed at contribuing to the improvement of cocoa tolerance against attack of *Phytophthora megakarya* with the use of aqueous and ethanolic extracts of pepper. The results obtain will allow to evaluate the effectiveness of the various pepper extracts on the *Phytophthora megakarya* isolates through *in vitro* and *in vivo* tests.

## MATERIALS AND METHODS

### Description of the study area

The study was conducted in the phytopathology laboratory of the Agricultural Research Institute for Development (IRAD), located at Nkolbisson a district in Yaounde found in the Mfoundi division in the Central region of Cameroon.

### Biological material

The plant material consisted of 4 months old leaves of eight hybrid genotypes from a hybrid family of cocoa (UPA134 X SNK13) and pods of clone SNK64 produced by IRAD Nkolbisson where the work took place as well as a variety of pepper (*Capsicum annuum*) obtained at a market in Yaounde. The fungal material consisted of two

*Phytophthora megakarya* strains isolated on mature pods naturally affected by black pod obtained both from the Awae locality in the Central Region and Mbanga locality in the Littoral region respectively

**Methodology**

***Isolation and characterization of Phytophthora megakarya***

Isolation of *Phytophthora megakarya*

Strain isolation was done according to the modified methodology of Rubini et al. (2005). Infected pods were collected from two agro-ecological zones with bimodal and monomodal rainfall and brought to the laboratory. The surfaces of the pods were previously washed with tap water and disinfected with 70% ethanol for 30 seconds in order to eliminate the microorganisms present on the cortex. The pods were then rinsed three times with sterile distilled water to remove any traces of disinfectant. The sampling was done at the level of growth front of the fungus. The superficial tissues were stripped with a sterile scalpel. 7mm sided pieces of cubic shape were removed under the cortical tissues of each pod. Collected fragments were cultured on agar medium contained in petri dishes. The incubation was done for 7 days for Oomycetes in the dark, in a tray respecting growth conditions .

**Purification of different strains**

Purification was carried out by transfer of colonies grown after 5 days in dishes containing PDA (Potato Dextrose Agar) and SPA (Small Pea Agar) culture media. The recovery of each colony in a dish was carried out three times successively. Incubation was carried out at a temperature of 26 °C for 10 days. This method was repeated until pure colonies were obtained.

**Macroscopic and microscopic observation and pathogenicity test**

Macroscopic observations were made after incubation 24 hours each day. After 14 days of incubation, the observation was made under a microscope using an identification key.

The pathogenicity test was performed to verify whether the strains obtained were *Phytohthora megakarya* strains. The apparently healthy pods harvested early in the morning were used for the pathogenicity test. They were washed with distilled water and disinfected with 70 ° alcohol for 3 minutes. Then, a cookie cutter of 0.5 cm of diameter was used to make a 1 cm deep hole in the center of each pod. Then, a fungal disk inoculum removed from a mycelial colony from 10 days old of culture was introduced into the opening made on the pods (Assiri et al., 2017).

**Test of the best growth substrate**

***Culture medium of SPA (Small Pea Agar)***

This culture medium is selective for the trapping and purification of *Phytohthora megakarya* (Huguenin and Boccas, 1971). On a scale, 70 g of pea were weighed and ground with a mortar until a paw was obtained. Then, a small amount of distilled water was added to make it liquid. After filtration, 15 g of agar and 250 mg of chloramphenicol were added to the solution and the volume was made up to 1000 ml with distilled water. After homogeneity, the medium was autoclaved at 115 ° C for 30 min.

***Culture medium of PDA (Potato-Dextrose-Agar)***

It is used for transplanting and collecting mushrooms. (Benhanou and Chet, 1996). During the preparation of the PDA culture medium, 200 g of potatoes previously washed and cut were weighed on a balance, and boiled for 45 minutes with distilled water on a hot plate. Then, the boiled potato solution was collected and introduced into a graduated Meyer Erlenmeyer. 15 g of agar and 20 g of dextrose were added to the solution. The volume was completed to 1000 ml with distilled water. After homogeneity, the medium was autoclaved at 115 ° C for 30 min.

**_In vitro_ evaluation of the effectiveness of the various pepper extracts on _Phytophthora megakarya_ isolates**

***Obtaining aqueous extracts (AQE) and ethanolic extracts (ETHE) from pepper in the laboratory***

The extracts were prepared according to the modified method of Nguessan et al., 2007. The work was carried out with two different extracts of pepper. The selected pepper variety was sorted, washed and oven-dried at 55 °C for 15 days (Koffi et al., 2014). The dried fruits were crushed in an electric mixer (3000 rev/min). The resulting mixture was sieved (mesh about 1-2 mm in diameter). The powder obtained was used to make the extracts. Two solvents (sterile distilled water and ethanol) were used. The aqueous extract was obtained by dissolving 40 g of powder in 400 ml of boiling distilled water and boiling for 15 minutes. The resulting homogenate was filtered through Whatman paper N° 2. The resulting filtrate was transferred to a vial and evaporation was done at 50 °C. The filtrate obtained constituted the total aqueous extract (AQE).

Ethanolic extract (ETHE) was obtained by dissolving 20 g of pepper powder in 200 ml of a 70% ethanol solution and then homogenized for 24 hours with the aid of a magnetic stirrer. After filtration on Whatman paper N° 2, the filtrate collected was evaporated in an oven at 50 °C. The resulting mixture constituted the ethanolic extract. These two extracts were tested separately on the growth of the different isolates.

**Preparation of the different concentrations of extracts**

***Dilutions made with the aqueous extract***

The stock solution of aqueous pepper extract was prepared from 40 g of pepper powder in 400 ml of distilled water. The mass concentration (C) of 0.1 g / ml was obtained. Four different concentrations (C0, C1, C2, C3) were obtained by dilution. The first concentration was C0 = 0.1 g / ml, representing the stock solution. The second concentration (C1 = 0.01 gm / l), obtained from 5 ml of the first concentration (C0) which were introduced into a tube containing 45 ml of distilled water for a final volume of 50 ml. The third concentration (C2 = 0.001 g / ml), obtained from 5 ml of C1 which were introduced into 45 ml of distilled water for a final volume of 50 ml. The fourth concentration (C3 = 0.0001 g / ml), obtained from 5 ml of C2 which were introduced into 45 ml of distilled water for a final volume of 50 ml.

***Dilutions made with the ethanolic extract***

The stock solution of ethanolic extract of pepper was prepared from 20 g of pepper powder in 200 ml of a 70% ethanol solution. The mass concentration (C) of 0.1 g / ml was obtained. Four different concentrations (C0, C1, C2, C3) were also obtained by dilution. The first concentration was C0 = 0.1 g / ml representing the stock solution. The second concentration (C1 = 0.01 g / ml), obtained from 5 ml of the first concentration (C0), which were introduced into a tube containing 45 ml of distilled water for a final volume of 50 ml. The third concentration (C2 = 0.001 g / ml), obtained from 5 ml of C1, which were introduced into 45 ml of distilled water for a final volume of 50 ml. The fourth concentration (C3 = 0.0001 g / ml), obtained from 5 ml of C2, which were introduced into 45 ml of distilled water for a final volume of 50 ml. The tubes containing the solutions were stored in aluminum foil to optimize the potency of its active ingredient (capsaicin) in the oven at 40 °C.

**Preparation of chemical fungicide or positive control**

Following the doses prescribed by the manufacturer, 20 g of the fungicide powder was diluted in 6.7 liters of distilled water at a mass concentration of 2.9 g / l.

**Amendments of pepper extracts to culture media and transplanting**

Under a horizontal laminar flow hood, various amendments were made to verify the effectiveness _in vitro_ culture of different extracts. After having prepared the extracts at

different concentrations, 5 ml of each concentration of extract were introduced respectively into the tubes containing 45 ml of culture media (PDA, SPA). These culture media were then poured into the Petri dishes of 90 mm diameter and inoculated with the randomly arranged isolates in a solidification and subculture incubator according to the Table 1. The diameter of the mycelium was measured 48 hours after incubation using a graduated ruler on each Petri dish. The level of infection was evaluated by calculating the area of necrosis according to the formula of Blaha and Lotode (1976).

Mycelial growth is estimated every 24 hours by measuring the average of the most dissimilar perpendicular diameters of each colony. Three replicates were performed for each concentration and for each isolate. The percentage of inhibition (PI) relative to the control is calculated according to the following formula of Mboussi et al. (2016).

**Fungicidal and fungistatic tests of different extracts**

The Petri dishes having the different extracts showing no growth of the mycelium after several weeks of incubation were selected. The explants of these Petri dishes were transferred into a new culture containing only essentially the PDA medium for observation.

***In vivo* evaluation of the effectiveness of pepper extracts on *Phytophthora megakarya***

**Leaf disc test**

***Leaf collections and record making***

Leaves of healthy green hybrid genotypes of approximately 4 months old were collected at 6:30 am (Tahi et al., 2007). The leaves of each plant were placed in each plastic bag numbered 1 to 8 into which were introduced a few drops of distilled water. In the laboratory, the sheets placed in bags were thoroughly washed with distilled water and stored in the dark until the next morning (Tahi, 2003). Very early of the next morning, after cutting them using a Cork Borer, we applied the treatments on each corresponding leaf disc.

***Treatments of leaf discs***

Four treatments (ETHE, AQE, positive control and negative control) were performed per bin for 8 hybrid genotypes and 10 replicates for each treatment (4 treatments × 10 repetitions × 8 genotypes) for a total of 320 leaf discs distributed in the 8 plastic tubs. These leaf discs were sprayed with extracts and chemical fungicide and kept moist. The 320 leaf discs were randomly arranged in up to 10 abaxial discs (Nyasse et al., 1995) and each bin contained a hybrid genotype for 4 treatments each having a row of 10 leaf discs on the wet paper towels in each bins with dimensions of 30 × 30 × 15 cm (length × width × height) numbered from 1 to 8.

**Preparation of the inoculum of *Phytophthora megakarya***

The preparation of the *Phytophthora megakarya* inoculum was made with 20-day-old isolates. To determine the number of spores produced by the strain used, a spore suspension was prepared by adding 5 ml of distilled water to the Petri dishes containing the isolates on PDA medium at 26 °C. Once the distilled water was added to the Petri dish, a Pastor pipette was used to scrape the surface to collect the resulting solution and to contain it in a beaker. The solution was then transferred to a refrigerator (4 °C) for 30 minutes and then in a dark place for one hour. This operation was performed to cause a thermal shock favorable to the release of zoospores of sporocysts. The Malassez cell was used to count the number of zoospores produced and released. The final concentration of zoospores in the inoculum was adjusted to $5.5 \times 10^5$ zoospores per ml at the objective × 10 (Nyasse et al., 1995).

**Infection of leaf discs**

The surface of the abbey leaf, through which natural infections occur more frequently was infected by depositing in its middle, using a micropipette, 10 microliters of zoospore suspension. The disks of each plant, placed in rows, were infected transversely to the rows so as to successively infect one disc per row (Nyasse et al., 1995) and to

randomize any effect of the zoospore batch on the different hybrid genotypes. The trays containing the infected leaf discs were sealed with a pane to maintain darkness in the interior and 100% relative humidity. Covered batches were placed in the laboratory in a dark, sterile room for a healthy disk incubation for 14 days, avoiding direct sunlight at a controlled temperature of approximately 26-28 °C using a air conditioner (Nyasse et al., 2002, tahi et al., 2007). The development of leaf symptoms was monitored over a two-week period and the disease severity index (SI) was calculated according to the following formula: (El Kaissoumi et al., 2016).

$$SI(\%) = \frac{\Sigma nb}{(N-1) \times T} \times 100$$

n = number of leaves for each degree of the scale;
b = degree of the arbitrary scale representing the severity;
N = number of degrees used in the scale;

The infection coefficient (IC) of the disease is determined according to the following equation (El Kaissoumi, 2016): IC = Severity x Incidence.

**Test on cocoa pods detached**

***Sample collection and processing of pods***

Healthy pods with clone SNK64 at physiological stage young pod, were collected in the field very early in the morning in disease-free areas, then taken to the laboratory where they were washed and placed in the plastic bins. The pods were divided into eight plastic tubs previously containing paper towel impregnated with distilled water to keep the moisture inside the bins and allow the development of *Phytophthora megakarya*. Four labeled pods were placed per tray and 3 replicates for each treatment. The three treatments (ethanolic extract, aqueous extract, chemical fungicide) were applied to 3 pods per tray and the fourth pod was used as a negative control. These bins were covered and left for 24 hours for the treatments to have their effect.

***Preparation of the inoculum and infection of the pods***

To determine the number of zoospores produced by the retained strain, a solution with zoospores of Oomycetes was prepared by removing the white powder containing the sporocyst with a spatula and introduced into a beaker containing 10 ml of distilled water. The solution was then transferred to a refrigerator (4 °C) for 30 minutes and then in a dark place for one hour. This operation was performed to cause a thermal shock favorable to the release of zoospores of sporocysts. Malassez's cell was used to count the number of zoospores released. The final concentration of zoospores in the inoculum was adjusted to $5.5 \times 10^5$ zoospores / ml.

The next morning, 20µl of suspension of zoospore solution were removed using a micropipette and deposited in the notches of each pod of 8 plastic tubs. These cuts were then covered with cotton soaked in distilled water. The bins were then maintained under relative humidity conditions for 14 days to promote and observe the development of *Phytophthora megakarya* from the Nyasse et al. 1995 scale.

***In vivo experimental device***

A complete randomized device was used. From 8 bins of pods, each contains 4 pods of clone SNK64 and from 8 bins of leaf dics, each contains 80 leaf discs for each of the 8 hybrid genotypes according to Table 2. Applications of water, aqueous extract, ethanolic extract, and chemical fungicide were sprayed on the eve of infection on each organ contained in the bins (one organ treatment with 03 replicates on the pods and 10 replicates on the leaves).

**Statistical analyzes**

The data obtained from the various tests of this study were compiled in the Microsoft Excel spreadsheet and analyzed using the XLSTAT 2014 software. The data underwent a general linear regression model with one and two-factor analysis of variance (ANOVA) followed by the Student Newman-Keuls (SNK) multiple comparison tests at the 5% threshold.

**Table 1:** *In vitro* experimental setup.

| Codes | Treatments | Concentrations (g/ml) | Repetitions × culture media (SPA, PDA) |
|---|---|---|---|
| **AQE** | Aqueous extract | $10^{-1}$ | 03×02 |
| | | $10^{-2}$ | 03×02 |
| | | $10^{-3}$ | 03×02 |
| | | $10^{-4}$ | 03×02 |
| **ETHE** | Ethanolic extract | $10^{-1}$ | 03×02 |
| | | $10^{-2}$ | 03×02 |
| | | $10^{-3}$ | 03×02 |
| | | $10^{-4}$ | 03×02 |
| **C+ (Positive control)** | Chemical fungicide | 2.7 | 03×02 |
| **C- (Negative control)** | Distilled water | 0 | 03×02 |

**Table 2:** Experimental device *in vivo*.

| Codes | Treatments | Organs | Concentrations |
|---|---|---|---|
| **AQE** | Aqueous extract | -04×03 (pods) | 0.1g/ml |
| | | -10×8 (leaf discs) | |
| **ETHE** | Ethanolic extract | -04×03 (pods) | 0.1g/ml |
| | | -10×8 (leaf discs) | |
| **C- (Negative control)** | Distilled water | -04×03 (pods) | - |
| | | -10×8 (leaf discs) | |
| **C+ (Positive control)** | Chemical fungicide | -04×03 (pods) | 2.9g/l |
| | | -10×8 (leaf discs) | |

## RESULTS

### *In vitro* evaluation of the effectiveness of the various pepper extracts on *Phytophthora megakarya* isolates

#### *Evaluation of the effectiveness with aqueous extract*

The results of Figure 1 showed the effect of the aqueous pepper extract on the *in vitro* growth of *Phytophthora megakarya*. These results (1a and 1d) show a complete inhibition of the growth of the mycelium in the presence of the aqueous extract and consequently, a total absence of the growth of the mycelium under *in vitro* conditions (0%) in the presence of the aqueous extract when the control at Day 8 is 100% of the development of the necrotic surface. These results (Figure 1) reveal an important difference between the necrotic surfaces observed in the different treatments and controls. Results (1c and 1f) also showed a complete inhibition of Oomycete in the presence of the chemical fungicide (0%) used as a positive control. By comparing the results (1a and 1d) with the others (1b and 1e) which represent the negative control, and where the

growth was normally carried out until the 7th day, the aqueous extract of the pepper has an inhibiting power on the growth of *Phytophthora megakarya* under *in vitro* conditions.

The results in Figure 2 showed the daily evolution of the necrotic surface of the Center strain under *in vitro* conditions in the presence of the aqueous extract at the different dilutions (0.1 g / ml, 0.01 g / ml, 0.001 g / ml and 0.0001 g / ml) of the aqueous pepper extract. No growth of the mycelium was observed in the presence of the aqueous extract irrespective of the dilution (concentration) of the aqueous extract of the pepper used. The same results were also observed in the presence of the positive control (d +) which is the chemical fungicide. The percentage of inhibition is therefore 100% irrespective of the dilution of aqueous extract. The only growth that occurred during this step was in the negative control (D0), where growth was complete after 7 days of incubation.

The results of the study of the Littoral strain (Figure 3) also showed the absence of mycelium growth at different dilutions (0.1 g / ml, 0.01 g / ml, 0.001 g / ml and 0.0001 g / ml) of the aqueous pepper extract as well as to the positive control. However, at the level of the negative control having media containing no extract, the growth was complete after 7 days of incubation.

**Evaluation of the efficiency with ethanolic extract in petri dish**

Results 4a, 4b and 4c of Figure 4 represent Central strain whereas results 4d, 4e and 4f represent Littoral strain. A few days after incubation, dishes (4c) and (4f) showed a production of spores on the whole surface of the petri dish. On the other hand, the dishes having the ethanolic extract (4b and 4e) slightly produced spores in relation to the concentrations of the ethanolic extract. However, the disches (4a) and (4d) containing the chemical fungicide (positive control) did not have any growth of the mycelium.

The graphs in Figure 5 and Figure 6 showed the effect of ethanolic pepper extract on the Central and Littoral strains of *Phytophthora megakarya* as a function of the necrotic surface ($cm^2$). For the dilutions d1, d2, d3 and d4 representing the concentrations of the ethanolic extract. The growth of the fungus depends on the concentration of the extract. The higher the concentration (d1 = 0.1 g / ml), the smaller the necrotic surface. However, at the d0 dilution (negative control), the growth of the mycelium is maximum (13.35 $cm^2$). Whereas at different concentrations of ethanolic extract, the growth of the mycelium is maximum when it reaches 6.5 $cm^2$ (Center) and 5.8 $cm^2$ (Littoral) which corresponds to a difference of 48.15% for Central strain and 43.44% for Littoral strain. These results reflect the inhibitory effect of the ethanolic extract on the growth of *Phytophthora megakarya* (Figures 5 and 6).

Results of Figure 6 also show the effect of ethanolic extract on the growth of Littoral strain. The results observed showed that there is no growth of the mycelium at the dilutions d1 and d2 (0 $cm^2$). At dilutions d3 and d4, a slight growth of the mycelium is observed. This slight growth is estimated as a function of the growth of the mycelium in the negative control. Between d0 and d3, the percentage of difference is 71.85%, while between d0 and d4 the difference is 53.03%. These percentages show a big difference between the negative control and the dilutions which allowed a slight growth of Oomycete.

**Inhibitory effect of pepper extracts in *in vitro* culture**

The results in Table 3 showed that (all two) both strains (Center and Littoral) of *Phytophthora megakarya* are sensitive to certain concentrations of ethanolic extracts and to all concentrations of aqueous extracts. Concentrations C1 and C2 inhibited (100%) the growth of the *P. megakarya* strain of the Center. The C1, C2, C3 and C4 concentrations of the ethanolic extract showed important differences for the two strains (Center and Littoral).

The variance analysis of the data showed a significant effect of the different dilutions of ethanolic extract applied *in vitro* on Central and Littoral Strains of *Phytophthora megakarya* (P <0.05). The results also showed significant differences in the ethanolic extract depending on the different dilutions in both *P. megakarya* strains (Figures 7a and 7b). In general, the aqueous extract treatments for all dilutions and ethanolic extract for 0.1 g / ml and 0.01 g / ml dilutions significantly (P <0.05) reduced Oomycete growth compared to negative control.

**Minimum inhibitory concentration at 50 and 90**

The results obtained show that the minimum inhibitory concentration (MIC) of the ethanolic extract varies according to the strains (Center and Littoral) whatever the level considered. At MIC 50, the MIC of the ethanolic extract is 0.0108 g / ml for the Littoral strain and 0.035 g / ml for the Central strain. At MIC 90, the MIC of the ethanol extract is 0.137 g / ml for the Littoral strain and 0.52 g / ml for the Central strain (Figure 8).

***In vivo* evaluation of the effectiveness of pepper extracts on *Phytophthora megakarya***

**Leaf disc test**

After treatment and infection of the leaf discs with a zoospore suspension at 5.5 × 10 5 per ml, the data were taken after 48 hours of incubation for 14 days (Figure 9).

Leaf discs that received no pre-infection treatment showed significant signs of necrosis at a very high percentage (95%). On the other hand, the discs which have received the application of the aqueous extract treatment (5%) and the chemical fungicide (0%) showed almost no sign of necrosis. However, the ethanolic treatment applied on the leaf discs allowed a slight development of the necrosis (25%) on one hand and on the other hand, the cell damaged of the leaves.

The shape of the different curves of the Oomycete attack index (Figure 10) is a function of the treatment applied to the leaf discs as a function of the period. The parameters were taken after 48 h of incubation for 14 days.

Treatment from the aqueous extract from day 2 to day 6 showed no development of necrosis. However, on the 8th day, the development was very weak (5%). A significant difference between the aqueous extract treatment and the negative control was observed during the 14 days (P <0.05). The treatment of leaf discs from the ethanolic extract showed signs of necrosis development. From the 8th day of taking parameters, a slight increase in the severity index was noted until the 14th day, representing 25% of the attack as a whole was observed. Chemical fungicide treatment did not develop foliar leaf necrosis (0%). On the other hand, the leaf discs having received no treatment application had a significant attack index of 5 after 14 days (100%) thus showing a total recovery of the leaf by the necrosis. These results also showed that the aqueous extract was very effective given the almost total absence of growth of *Phytophthora megakarya* on hybrid genotypes. In fact, the average attack index for all bins was 0.05 versus 2.7 for the average of the negative control. Treatment with ethanolic extract, however, showed a slight inhibition on the growth of the fungus, but still less important than the result obtained with the treatment of the negative control (Figure 10).

**Test on pods detached in the laboratory**

The mature pods were pretreated according to the treatment and each of them was infected with the *Phytophthora megakarya* strain and after 48h, the parameters were taken. Three days after infection of the pods, points of infection were observed (Figure 11a) on untreated pods. While in the same bin, pods pretreated with ethanolic extract 4 days after infection showed signs of black pod desease but no spore development on the pods surface (Figure 11b). On the other hand, on the same date, the pods pretreated with aqueous extract did not develop any sign of infection. (Figure 11b).

Observations made on the 7th day after infection for untreated pods and those that received the application of the ethanolic extract, developed slightly greater signs of necrosis on the surface of these pods (Figure 11b). The pods on the 14th day had an even more important aspect. Untreated pods were invaded by symptoms of black pod desease followed by spore formation on the pods surface. (Figure 11c).

The application of extracts treatments on the pods allowed to notice the antifungal power of these extracts. Table 4 illustrates the effectiveness of all treatments on pods from the averages reported. This table shows a significant difference between treatments (aqueous extract and ethanol extract) and controls (distilled water and chemical fungicide). The treatment with the aqueous extract is not significantly different from that of the chemical fungicide ($P<0.05$).

The pods on which the aqueous extract was applied from day 2 to day 8 showed no evidence of necrosis development. However, from the 10th day, a slight development of the necrosis representing 5% of the attack appeared. Treatment with ethanolic extract also showed a significant difference from the control (Figure 12). At day 14, the necrosis attack index is 1.2 on pods pretreated with ethanolic extract when it reaches 5.2 on pods treated with distilled water.

The application of the aqueous pepper extract to the pods had a significant effect as a function of days ($P<0.05$). From day 2 to day 14, no evidence of necrosis development was observed on the surface of infected pods. The treatment of the pods with the ethanolic extract also showed a significant difference compared to the negative control at the 5% threshold. From day 2 to day 14, there was a slight sign of necrosis ranging from 0.05 to 1.3. The average attack index estimated for the ethanolic extract was 0.196. The application of the ethanolic extract did not allow the formation of spores on the surface of the pods, but rather a brown patch that developed on the pod. Untreated pods showed significant signs of necrosis from day 2 to day 14. The estimated average for control pods was 2.304.

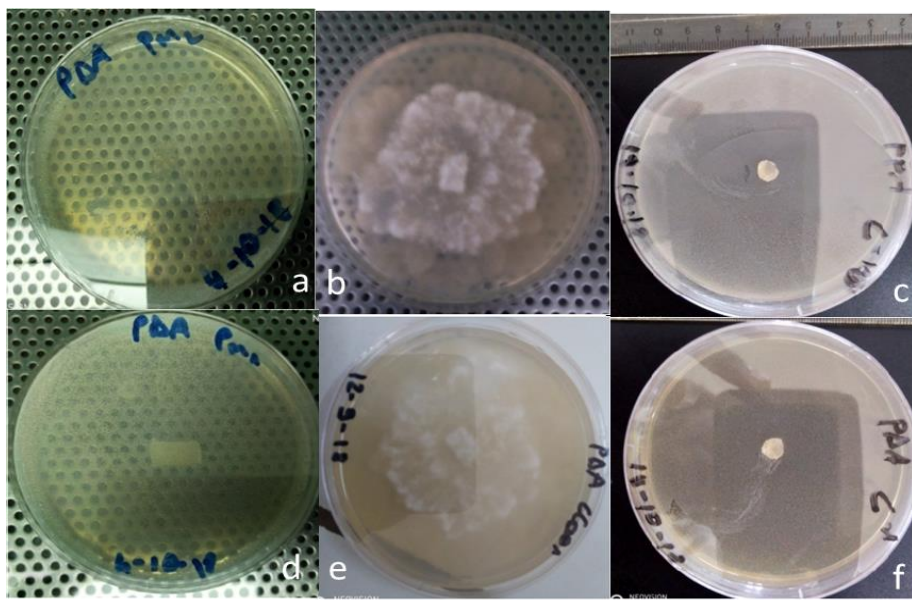



**Figure 1**: Petri dishes after 7 days: (a) and (d) petri dishes containing positive control; (b) and (e) negative control petri dishes; (c) and (f) Petri dishes containing aqueous extract.

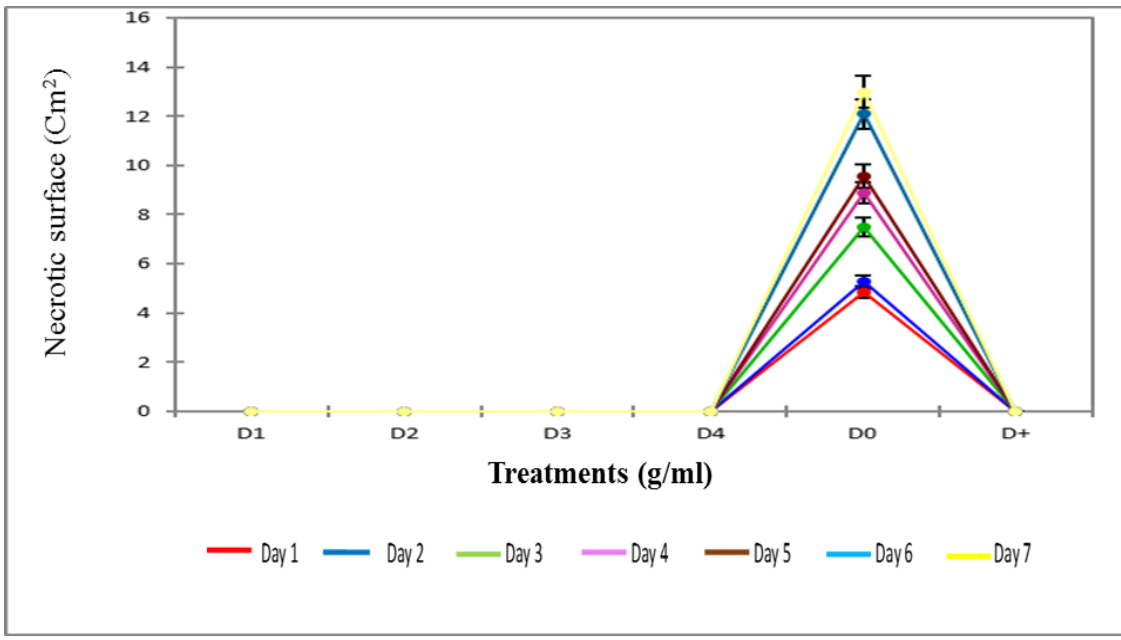



**Figure 2:** Radial growth of *Phytophthora megakarya*, Central strain in the presence of treatment doses with aqueous extract of pepper and controls (C- and C+).

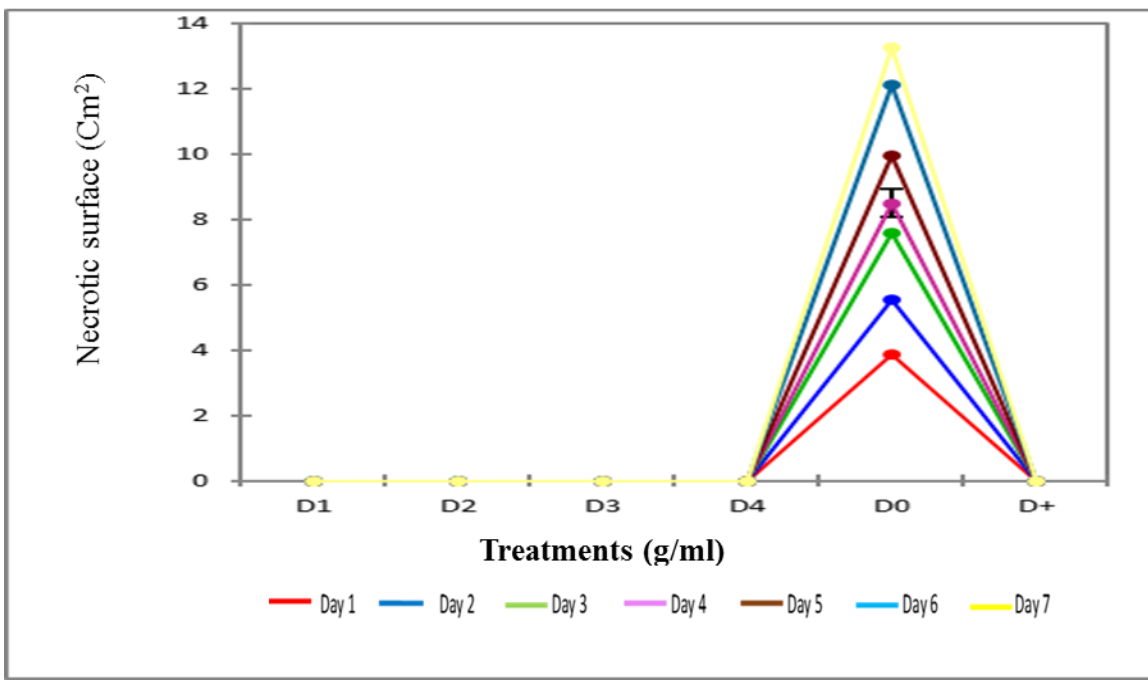



**Figure 3:** Radial growth of *Phytophthora megakarya*, Littoral strain in the presence of aqueous extract treatment doses and controls (C- and C+).

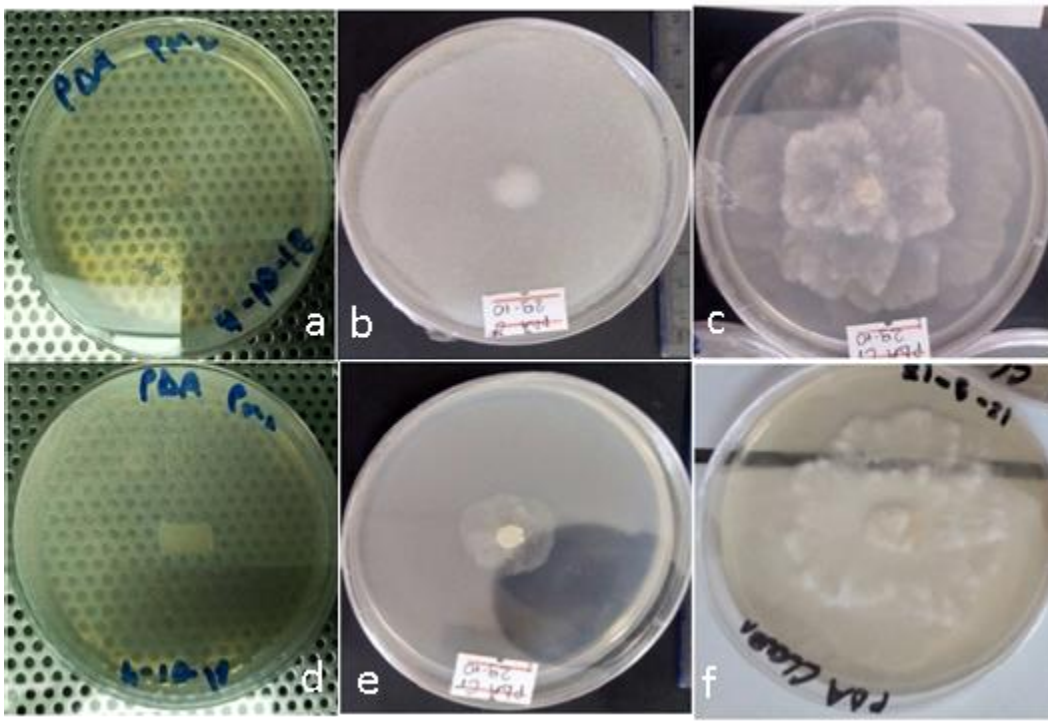



**Figure 4:** Petri dishes after 7 days of culture in the presence of ethanolic extract of pepper: (a) and (d) petri dishes containing positive control; (b) and (e) petri dishes containing ethanolic extract; (c) and (f) Petri dishes containing negative control.

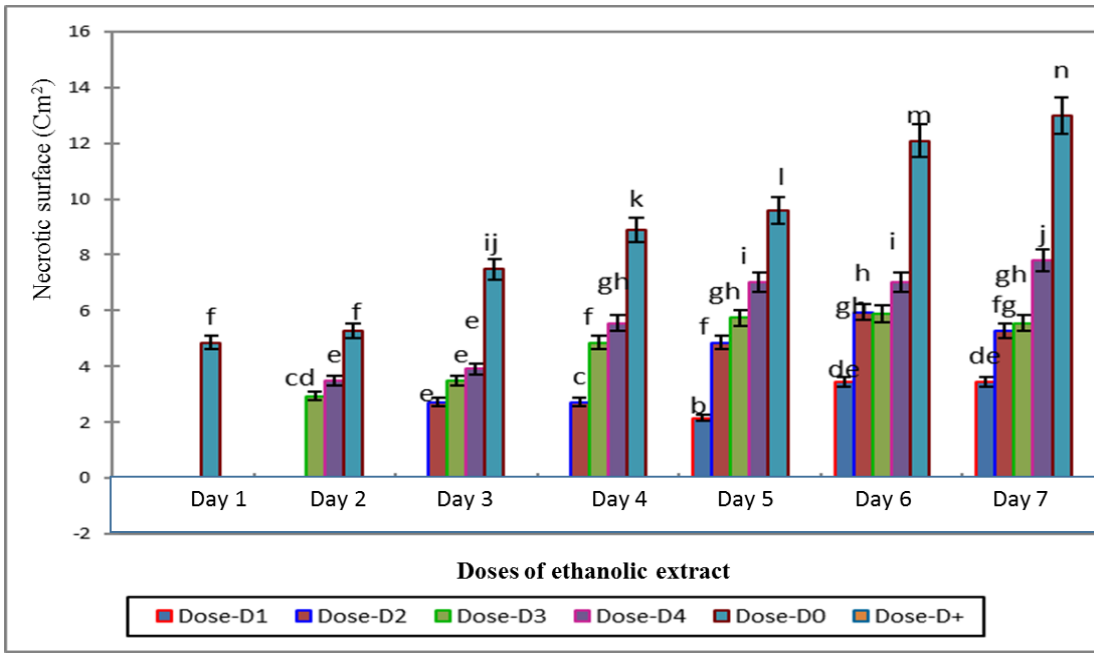



**Figure 5:** Daily radial growth of *Phytophthora megakarya* from the Central strain in the presence of ethanolic extract. Values of the same parameter with different letters are significantly different at P <0.05.

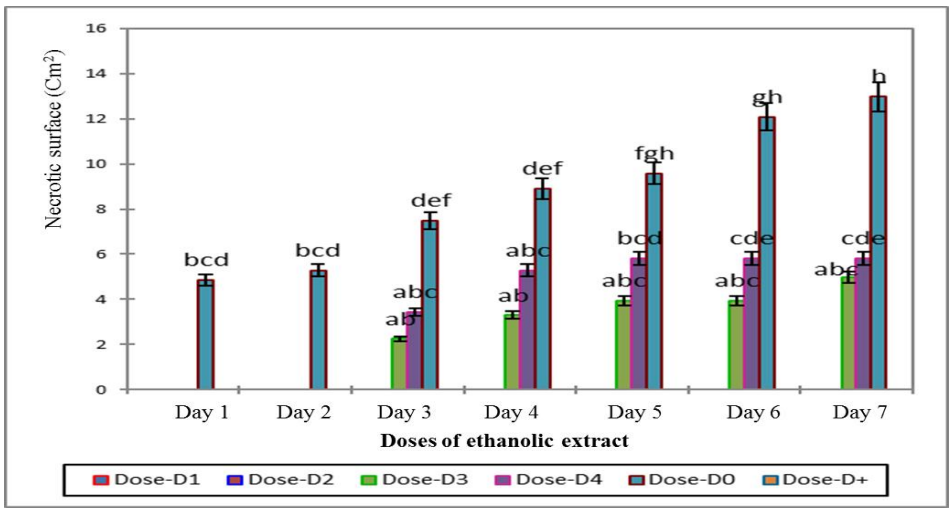
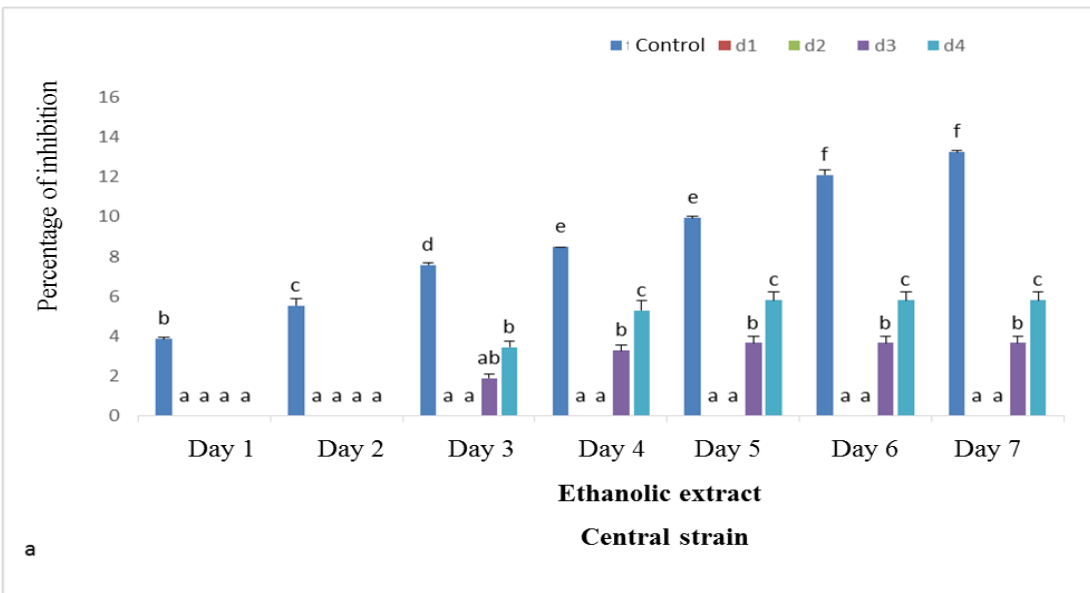



**Figure 6:** Daily radial growth of *Phytophthora megakarya* from Littoral strain in the presence of ethanolic extract. The values of the same parameter with different letters are significantly different at P <0.05.

**Table 3:** Percentage inhibition at different concentrations.

| | Central strain | | Littoral strain | |
|---|---|---|---|---|
| **Concentrations (g/ml)** | **ETHE** | **AQE** | **ETHE** | **AQE** |
| **$C_1$ (0.1)** | 100 | 100 | 89.33 | 100 |
| **$C_2$ (0.01)** | 100 | 100 | 63.35 | 100 |
| **$C_3$ (0.001)** | 70.22 | 100 | 54.39 | 100 |
| **$C_4$ (0.0001)** | 57.54 | 100 | 42.86 | 100 |
| **Control** | 0 | 0 | 0 | 0 |



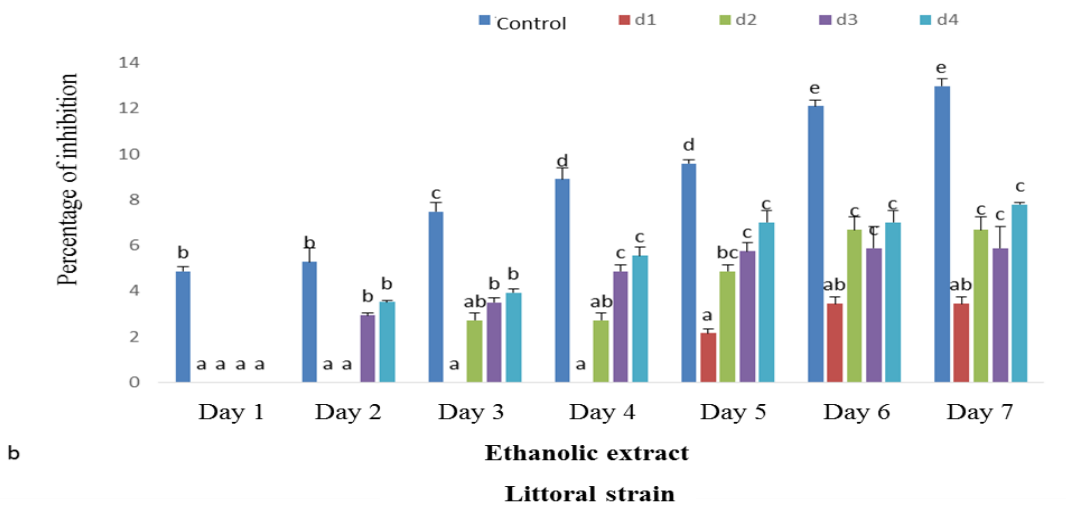



**Figure 7:** Percentages of inhibition of ethanolic extracts of both strains of *Phytophthora megakarya*.

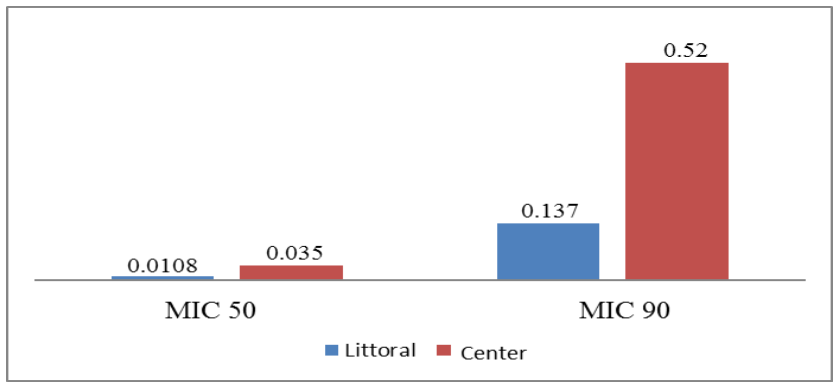



**Figure 8**: Ethanolic extract inhibitory concentrations.

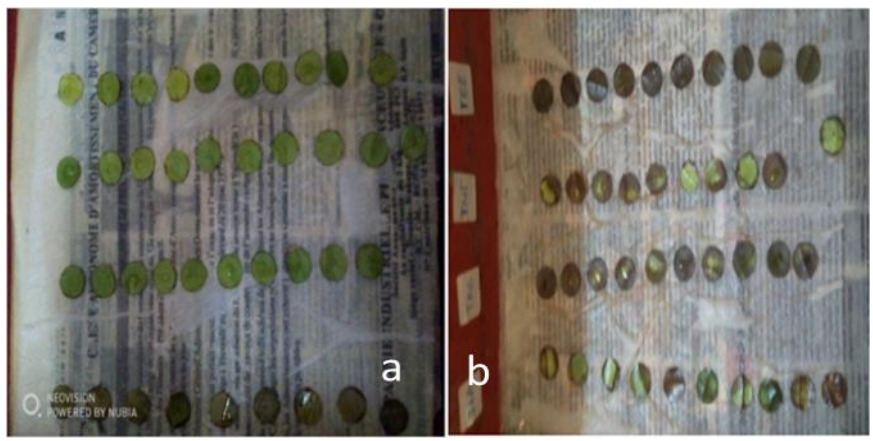



**Figure 9:** Infected leaf discs: (a) one day after infection; (b) 14 days after infection.

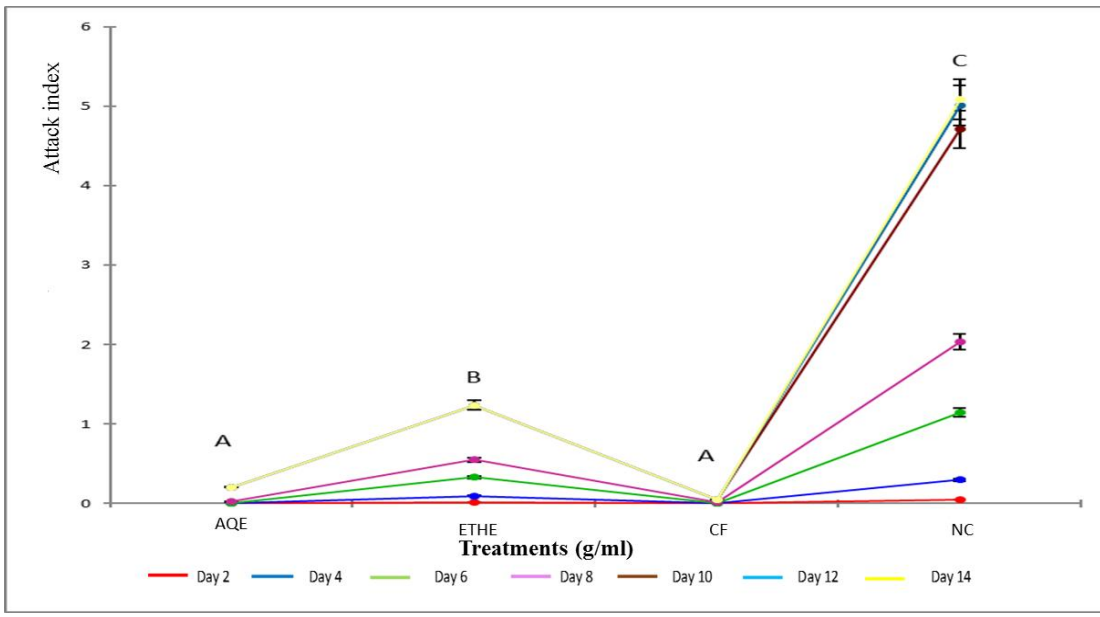



**Figure 10:** Average growth of leaf disc necrosis for four treatments: (AQE) Aqueous extract treatment; (ETHE) Ethanolic extract treatment; (CF) chemical fungicide or positive control treatment; (NC) negative control.


**Figure 11**: Containers containing infected pods: (a) 3 days after infection; (b) 7 days after infection; (c) 14 days after infection.

**Table 4:** *Phytophthora megakarya* pod attack index according to treatments.

| Modalities | Estimated averages |
|---|---|
| **AQE** | 0.036a |
| **CF** | 0.196a |
| **ETHE** | 1.750b |
| **Control** | 2.304c |

The values of the same parameter with different letters are significantly different at P <0.05 according to the Student Newman-Keuls test.

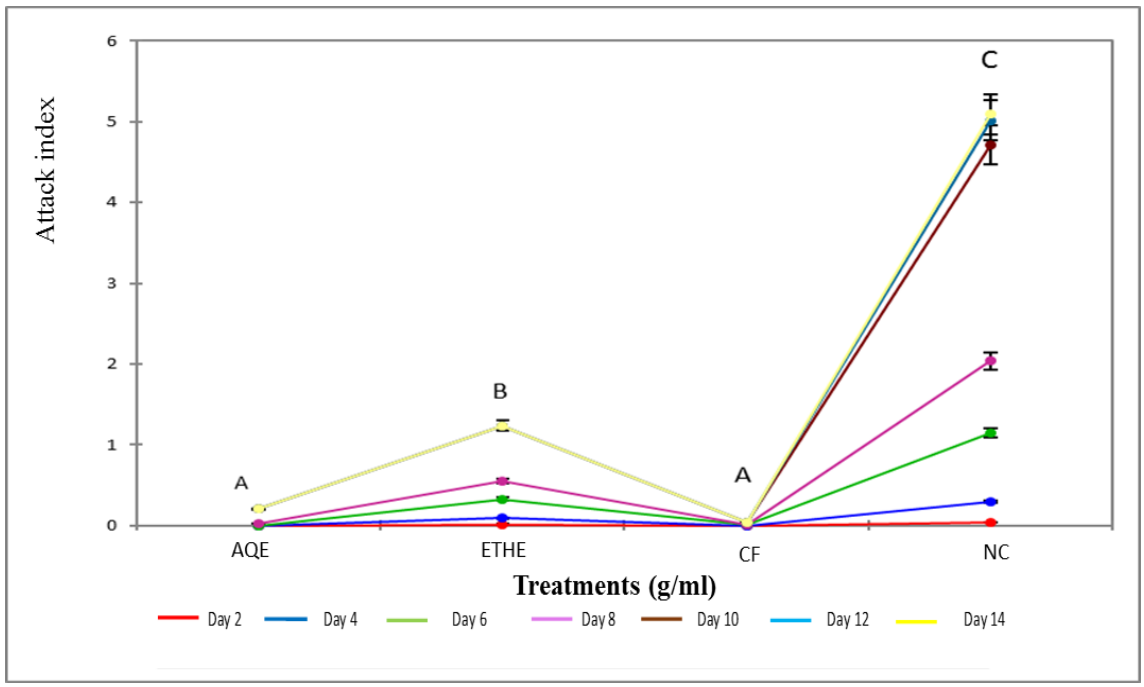



**Figure 12:** Average growth of pod fecrosis for four treatments: (AQE) aqueous extract treatment; (ETHE) Ethanolic extract treatment; (CF) chemical fungicide or positive control treatment; (NC) negative control.

## DISCUSSION

In order to make a contribution to reduce problems caused by *Phytophthora megakarya*, pathogen of black pod desease on cacao (*T. cacao*), the preparation and use of two extracts of pepper (*Capsicum annuum*) have been performed under *in vitro* and *in vivo* conditions to evaluate their antifungal effect on the causative agent of black pod desease.

### Evaluation of the antifungal activity of different doses of pepper extracts on *Phytophthora megakarya* isolates *in vitro*

The evaluation of the antifungal activity of pepper extract doses on *Phytophthora megakarya* isolates revealed the best applied concentration and the best pepper extract against *Phytophthora megakarya* after 7 days under *in vitro* conditions.

### Growth of the two strains of *Phytophthora megakarya* on SPA and PDA media

The first observation made is the radial growth of *Phytophthora megakarya* on PDA medium compared to SPA medium. The two isolates (Center and Littoral) of *Phytophthora megakarya* grow, but denser and faster on PDA medium. At day 8, the growth of Oomycete was 55.8% for both strains on SPA medium when it reached 97% on PDA medium. These results showed that PDA medium is the best culture medium for *Phytophthora megakarya*. These results are due to the presence of nutrients that would allow very good growth for Oomycete isolates in the PDA culture medium. This PDA medium is also rich in energy element in relation to the presence of the carbon source (dextrose) that would promote the rapid growth of this pathogen. These results corroborate the work of Kra et al. (2009) who showed under *in vitro* culture that PDA

medium was the best medium for the growth of *Fusarium spp*. However, Coulibaly et al. (2013) in their work on the morphological and molecular characterization of *Phytophthora spp* isolates from cocoa orchards in Côte d'Ivoire found that the suitable medium for the growth of *Phytophthora spp* was the SPA medium after testing several isolates. The results obtained are also different from those obtained by Mpika et al. (2009) who showed that the best medium for the growth of *Phytophthora spp* was the SPA medium during their work on the inhibition of *Phytophthora palmivora*, a black pod desease agent of cocoa in Côte d'Ivoire by the antagonist agent *Trichoderma sp* under *in vitro* culture.

**Test of the antifungal activity of *C. annuum* extracts**

The antifungal activity test with pepper extract was carried out on two isolates (Center and Littoral). Four different concentrations (g / ml) (C1 = 0.1, C2 = 0.01, C3 = 0.001, C4 = 0.0001) were used for each of the two extracts. Statistical analysis revealed that the effect of medium concentrations in pepper extract was significant at the 5% threshold; these results are similar to those of Fondio et al. (2015) who studied the agronomic and health behavior of new pepper lines (*Capsicum sp*) in southern Côte d'Ivoire. Their work has highlighted the fungicidal and antibacterial potency of pepper against pests. These results are also similar to those obtained by Fening et al. (2014) who evaluated the effectiveness of pepper in controlling diseases of *Brassica oleraceae* L., and *Phaseolus vulgaris* L. in two agro-ecological zones in Ghana. Their work has shown that the aqueous pepper extract used under *in vitro* culture significantly reduces the action of pests attacking natural enemies on the seedlings used.

In addition, the ethanolic extract of pepper under *in vitro* and *in vivo* cultures has a weak action on *Phytophthora megakarya* compared to aqueous extract which acts considerably to all the concentrations used. However, the work of Koffi et al. (2014) on the activity of the extracts of six varieties of peppers (*Capsicum*) used in Côte d'Ivoire have shown that the use of ethanolic extract of *Capsicum* have a higher antifungal activity than the aqueous extracts. The absence of the development of the mycelium in the Petri dishes containing the extracts is due to the fungicidal power that the pepper possesses through its active ingredient which is Capsaicin. The work of Kouassi (2012) has shown that *Capsicum annuum* and *Capsicum frutescens* varieties contain alkaloids, flavonoids, tannins, steroids and polyphenols with antimicrobial properties.

**Evaluation of inhibition percentages and MIC**

Based on inhibition percentages of pepper extracts (ethanolic and aqueous) on the growth of *Phytophthora megakarya*, it was observed that the aqueous extract significantly reduced mycelium growth compared to the negative control for all concentrations of aqueous extract (100%). However, with the ethanolic extract, variations in inhibition percentages are observed considering the concentrations and the source of the isolate. The Littoral strain showed at concentrations 0.1 g / ml; 0.01 g / ml; 0.001 g / ml and 0.0001 g / ml, the percentages of inhibitions respectively 89.33%; 63.35%; 54.39%; 42.86%. On the other hand, the Central strain showed complete inhibition (100%) at the first two concentrations (0.1 g / ml and 0.01 g / ml) and respective percentages of inhibition of 70.22% and 57.54% at concentrations 0.001 g / ml and 0.0001 g / ml. These results show that the higher the concentration of ethanolic extract, the greater the inhibitory effect. However, the percentages of inhibition obtained with the ethanolic extract is not greater than the percentages of inhibition obtained with the aqueous extract whatever concentration used. These results are different from those obtained by Traoré et al. (2012) who showed that ethanolic extracts of the same plant species are more active than the

aqueous extracts when the concentration is important during their work on the search for antifungal and antibacterial activities of *Annoma senegalen* leaves. Moreover, these results are similar to those obtained by Zouaoui et al. (2018) who worked on the contribution to the study of the antifungal power of the seeds of *Chenopodium quinoa* Wild against two phytopathogenic fungi *Pyrenophora tritici-repentis* and *Rhynchosporium secalis*. These authors, during their work, showed that the antifungal effect of plant extracts would be related to the previously used concentrations.

**Evaluation of the effect of pepper extracts on leaf discs**

The antifungal activity of pepper extracts (aqueous and ethanolic) was tested by evaluating the development of necrosis caused by strains of *Phytophthora megakarya* on leaf discs in the laboratory. Growth of *Phytophthora megakarya* occurred on control leaf discs. The *Phytophthora megakarya* isolates tested can be considered as pathogenic on *Theobroma cacao* plants (Minyaka et al., 2017). The isolates were able to penetrate the leaf cells and colonize them by inducing symptoms specific to *Phytophthora megakarya*. These results are in agreement with those of Djocgoue et al. (2010), Efombagn et al. (2011) and Ondobo et al. (2014) who studied the development of necrosis on pods and leaves of this same plant. The evolution of *Phytophthora megakarya* necrosis on leaf discs of hybrid families showed a progressive increase in the severity of the disease and foliar alterations in the untreated control as a function of time (95% infected). The evaluation of the necrosis showed a disease attack index of less than 0.2 in the presence of aqueous extract and less than 1.2 in the presence of ethanolic extract. These low average foliar sensitivity values of *Phytophthora megakarya* compared to the control reflect the inhibitory action of pepper extracts. These extracts significantly reduce the size and frequency of necrotic lesions due to Oomycete. These studies are similar to those obtained by Bowers et al. (2001) and Mpika et al. (2009), who showed that the use of antagonists such as *Trichoderma sp* reduces the development of the disease caused by the pathogen. The use of pepper would stimulate the defense mechanism of the plant and therefore enhance resistance to penetration and spread of the pathogen. Similar results were obtained through the work of Bigirimana et al. (1997), Howell et al. (2000), Sid Ahmed et al. (2000) and Harman et al. (2004) respectively in pre-infected bean, cotton, pepper and maize of *T. virens* and *T. harzianum* and attacked by *Phytophthora sp*. In addition, 40% of leaf disks treated with ethanolic extract had the wall of their cells destroyed. These results would be due to the effect of alcohol that would have been used in large quantities on the leaves.

**Evaluation of the effect of pepper extracts on pods**

The antifungal activity of aqueous and ethanolic pepper extract was observed on pods. The results revealed heterogeneity on the attack index of the action of each treatment (aqueous extract, ethanolic extract, distilled water, chemical fungicide). This difference is due to the ability of each extract to produce the active ingredient responsible for the antifungal properties of pepper as confirmed by the work of Koffi et al. (2014) who worked on the *in vitro* evaluation of the antifungal activity of three extracts of pepper (*Capsicum*) used in Côte d'Ivoire (aqueous extract, 70% ethanolic and acetal) on the growth of *Penicillium sp*. *Fusarium sp., Alternaria sp., Aspergillus flavus and Aspergillus niger*. Treatment with the aqueous extract significantly reduced ($P<0.05$) the attack of *Phytophthora megakarya* (5% attack) on pods compared to negative control pods. This reduction is due to the direct contact between the aqueous pepper extract and Oomycete. Capsaicin of pepper, would certainly act by interaction on several cellular processes of Oomycete, such as respiration, permeability, and cell division and would therefore cause a fatal effect to the

germination of spores (sporocyst and zoospores) which are at the origin of epidemics. These observations help to understand the effect of the extracts used to reduce black pod desease due to *Phytophthora megakarya* as confirmed by Pohe et al., 2012 who worked on the action of cupric salts and the periodicity of their application on the black pod desease in Côte d'Ivoire.

**Conclusion**

The objective of this work was to evaluate the antifungal activity of two pepper (*Capsicum annuum*) extracts (aqueous and ethanolic) in *Theobroma cacao* against the pathogen *Phytophthora megakarya*. From the results obtained, the leaf disc tests showed an attack index greater than 5 for the negative control, when it reached 1.2 for the ethanolic treatment and 0.1 for the aqueous extract. These results showed that the aqueous and ethanolic pepper extracts have antifungal power on foliar discs of *Theobroma cacao* infected with *Phytophthora megakarya*. On pods, *Phytophthora megakarya* has also proved to be sensitive to the application of pepper extracts. This test showed a significant difference between the ethanolic and aqueous extracts of pepper and the negative control. The Averages of attack observed for each treatment were 2.304 (negative control), 0.196 (aqueous extract), 1.750 (ethanolic extract) and 0.036 (chemical fungicide). The aqueous pepper extract could therefore be used as a biofungicide against *Phytophthora megakarya*, the causal agent of black pod desease in Cameroon.

**COMPETING INTERESTS**

The authors have the honor to inform the editorial team that there is no conflict of interest in this work.

**AUTHORS' CONTRIBUTIONS**

CS and AVTI designed the study; RNA, VL and SAN-N collected samples; LCNT performed experiment, analysed result and wrote the first draft of manuscript; PFD critically review the first draft. All authors read and made input to the final draft.

**ACKNOWLEDGMENTS**

The authors would like to thank the General Director of the Institute of Agricultural Research for Development (IRAD) of Nkolbisson for allowing this research work to be done in the phytopathology laboratory.

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