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Anti-Fungal Activities of *Jatropha curcas* Seeds Extracts against *Cercospora malayensis* Causative Agent of Sigatoka of Okra Leaves

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ABSTRACT

Sigatoka caused by *Cercospora malayensis* is one of the fungal diseases that causes significant yield losses on okra. The aim of this work was to evaluate the antifungal activity of organic and aqueous extracts of *Jatropha curcas* seeds on the *in-vitro* growth of *C. malayensis*. Six treatments were used: Four extracts: acetone, methanol, ethyl acetate and aqueous; one negative control (0 µl/ml) and one positive control (3,33 g/l). For each extract, five concentrations were applied (7,5 µl/ml, 15µl/ml, 30µl/ml, 60 µl/ml and 120µl/ml). The radial growth inhibition test was carried out, the MIC_{50}; MIC_{90} and correlations test between concentration and percentage of inhibition were determined. The results showed that the various organic extracts had an inhibitory effect on the radial growth of the strain at the concentration of 120 µl/ml. As for the aqueous extract, the inhibitory effect was obtained from the lowest concentration (0.7 µl/ml). These extracts have an efficient similarity to that of the synthetic pesticide. There were positive correlations (0,7 to 0,9) between the concentrations of different extracts and the percentages of inhibition which revealed that fungal growth inhibition was a function of extracts concentration applied. The low values (4.08, 3.50 and 3.41) of the MIC_{50} obtained, showed the effectiveness of the different extracts. Organic and aqueous extracts of *J. curcas* seeds presented a fungicidal potential that could be exploited in the integrated pest management of okro.

I. INTRODUCTION

Okra (*Abelmoschus esculentus*) is a flowering plant of the tropical region belonging to the family of Malvaceae originated from Africa. With an average annual production of 3 355 000 t/yr, India is the first producer and accounts for72 % of the world's production.

In Africa, the highest production comes from Nigeria, Ghana and Benin. In Cameroon, all the regions offer climatic conditions favorable for the cultivation of okra (Zonckeng and *al*., 2004). Nevertheless, there exist areas of high production and commercialization potentials: the Far North (3.7t/ha), the Centre (1.9t/ha) and the West Regions (1.1t/ha). According to the report of the Ministry of Agriculture (Cameroon), more and more young people are starting to colonize new lands to cultivate okra resulting in an increase in production (Ngeulieu, 2010). Okra is one of the most important traditional plants that can be found in all African markets (INERA / BF 2001, Nguelieu 2010). Okra is subjected to intensive production system in rural and urban agriculture. *Abelmoschus esculentus* has an important nutritional value that is far behind that of carrot but better than that of tomato (Sawadogo and *al*., 2006). It is grown throughout Africa, for its fruit (eaten as a sauce, fried like spinach). The mucilage of okra is used to increase blood volume, the leaves sometimes served as, poultices and are used for their emollient and sudorific properties, and in the treatment of dysuria (Siemonsma and Kouamé, 2004). Okra seeds can be used as coffee substitute or as a flocculating agent for water purification (Argawal and *al*., 2003, Siemonsma and Kouamé 2004, Okigbo 1975, Nguelieu 2010).

Despite its multiple uses, its proven nutritional value, its financial value and renewed interest in market gardening in general. Okra production in Cameroon, in particular, is still very low (Sawadogo and *al*., 2006 Medagam and *al*., 2012); (Ngeulieu, 2010). The major causes of this insufficient production are among others, the low availability of improved varieties in the market adapted to the conditions of the hot and humid agro-ecological zone, as well as the presence of diseases and pests that can cause significant drop in its production (Dubey and Bhagat, 1998, Ali and Hossain, 2000).

The pathogen *Cercospora malayensis* which causes Sigatoka is identified as responsible for the colossal losses in okra production.

To cope with these problems and to guarantee a high production, some countries have opted for the use of synthetic phytosanitary products (fungicides, nematicidal insecticides). The

high use of these products is considered a prerequisite for the success of a rapid agricultural development strategy. These products are excessively expensive and sometimes unavailable to local farmers. Due to their toxicological properties, they constitute a real hazard and are currently considered among the main environmental pollutants with toxic residues that are responsible for many human diseases (Sanderson *et al*., 2002, Perera and *al*., 2005, Watanabe-Akanuma and *al*., 2005).

In the search for more effective, less costly, non-polluting and human health-friendly plant protection alternatives, biological control with the use of natural substances of plant origin is of interest in the protection of field crops against diseases and pests of okra plant. The insecticidal and fungicidal properties of some plants have been demonstrated by several studies (Spadaro and *al*., 2001, Janisiewicz and *al*., 2001, Mboussi and *al*., 2016). Like most biodegradable fungicidal products, seeds of *Jatropha curcas* appear to have a high fungicidal potential against certain crop pests. In the framework of this study, the general objective was to test the fungicidal effect of the organic (ethyl acetate, acetone) and aqueous extracts of *Jatropha curcas* seeds on the strain of *Cercospora malayensis in vitro.*

MATERIALS AND METHODS

Plant material

The plant material consisted essentially of *Jatropha curcas* seeds harvested in the Centre Region of Cameroon.

Fungal material

The fungal material consisted of a purified strain of *Cercospora malayensis.*

Other materials

Many other materials were used for the realization of this work like organic solvents (acetone, ethyl acetate, methanol), Petri dishes, host, Erlenmeyer, crushing machine.

Methods

Extraction of pure strains of *Cercospora malayensis*

Pure strains were isolated from infected okra leaves showing symptoms of the disease. The leaves were harvested in the field and then taken to the laboratory where they were washed

several times with tap water, disinfected with a cotton soaked in 95% alcohol and then flame dried. Using a scalpel, the infected parts of the leaf (bearing the necroses) were removed and incubated in petri dishes containing PDA culture medium. The petri dish was closed, sealed with food film and incubated in a culture chamber at 22-24 °C under a 12h photoperiod. Mycelia grew from the explant and reached sufficient growth after 5 days, ready for purification. Purification was carried out by successive subcultures of an agar fragment taken from the growth front of mycelium on PDA medium. This operation was repeated several times until pure cultures were obtained that will be kept in pill containers, containing sterile distilled water (Ondo 2006, Nyassé 1992). The identification of the pathogen *Cercospora malayensis* was done through the observation of conidia on the microscope and an identification key (Vaz 1987, Hsieh and Goh 1990).

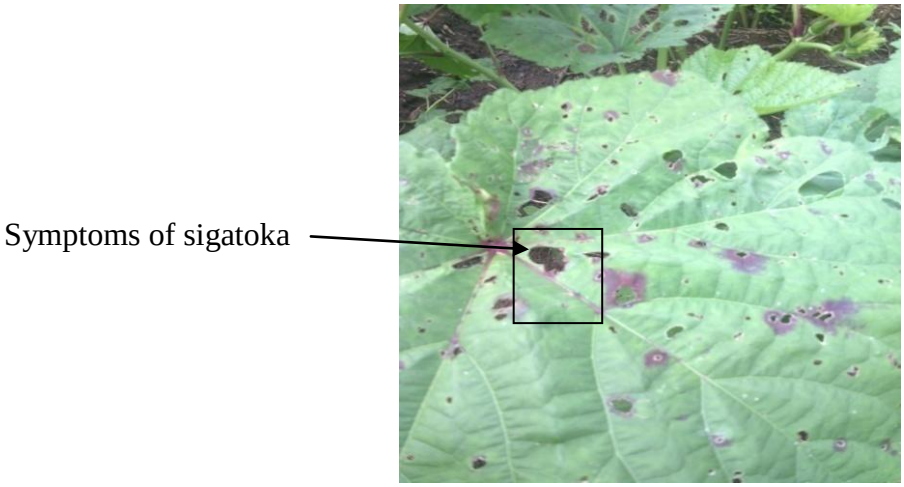


Fig.1.Characteristic symptoms of sigatoka caused by *Cercospora malayensis*

Preparation of PDA medium

For the preparation of the PDA, 200 g of Irish potatoes were cooked (cooking temperature) in water for 30 minutes. The boiled potatoes were pressed in filter paper and the juice collected in 1000ml beaker. 15g of agar and 15g of dextrose were added. The mixture was completed with distilled water up to 1000 ml and boiled on a hot plate equipped with a magnetic stirrer. The pH was adjusted to 6.0 if necessary. The solution obtained was sterilized in autoclave for 20 minutes at 120 °C and stored in a refrigerator. This medium was used for the culture of pure strains and growth inhibition tests of *C. malayensis*.

Preparation of different *Jatropha curcas* seed extracts

The mature fruits of *Jatropha curcas* were harvested or picked under these plants in different parts of the city of Makenene in the Centre Region of Cameroon. The fruits were pulped and the seeds obtained were dried at room temperature in the phytopathology laboratory of the University of Yaoundé I for 3 to 4 weeks. The seeds were cleared of their integuments, then dried. The dried seeds were crushed with a manual grinding machine of brand "Victoria". 500 g of seed powder was weighed using a precision scale and macerated in 2 liters of solvent for 72 hours (Stoll, 1994). The solute solvent mixture was transferred into the sonicator to maximize extraction. After filtering with the filter paper, the solution was transferred inside the rota-vapour for the separation of the solvent from the extractable compounds. The latter was then conserved in a refrigerated (4 °C) in a refrigerator for subsequent use. The aqueous extract was obtained by maceration of 500 g of seed powder in 1000 ml of distilled water for at least 24 hours.

Preparation of different doses of extracts to be tested in the laboratory

A stock solution of 500 µl/ml was first prepared by mixing 1 ml of pure extract with 0.3 ml of sterile distilled water and 0.7 ml of ethyl alcohol at 70 °. The different concentrations (7.5, 15, 30, 60 and 120 µl/ml) were prepared by successively withdrawing 0.45; 0.9; 1.8; 3.6 and 7.2 ml of this stock solution and adding respectively 29.55; 29.1; 28.2; 26.4; 22.8 ml of PDA for a final volume of 30 ml each. This final volume obtained was poured into Petri dishes of 90 mm, 10 ml each. These volumes were obtained using the formula (CiVi = CfVf) (Gata-gonçalves, 2001). For the negative controls, a solution of 10 ml of medium was directly poured into each Petri dish.

The preparation of the synthetic fungicide enriched medium (Monchamp) complied with the manufacturer's formulation doses of 3.33 g / l. For this, a stock solution of 50 mg/ml was first prepared by mixing 500 mg with sterile distilled water, for a final volume of 10 ml. A volume of 2 ml was taken from the stock solution and mixed with 28 ml of PDA medium for a final volume of 30 ml. These proportions were obtained using the relationship CiVi = CfVf.

For the aqueous extracts, a stock solution of 250 mg/ml was previously prepared by macerating 500 g of *Jatropha curcas* seeds in 2l of sterile distilled water for at least 12 hours. Culture media of 7.5; 15; 30; 60 and 120 µl/ml were prepared by successively withdrawing 0.9; 1.8; 3.6; 7.2 and 14.4 ml of the stock solution and adding 29.1; 28.2; 26.4; 22.8 and 15.6

ml of PDA respectively for a final volume of 30 ml each. These volumes were used using the formula CiVi = CfVf.

Evaluation of radial growth

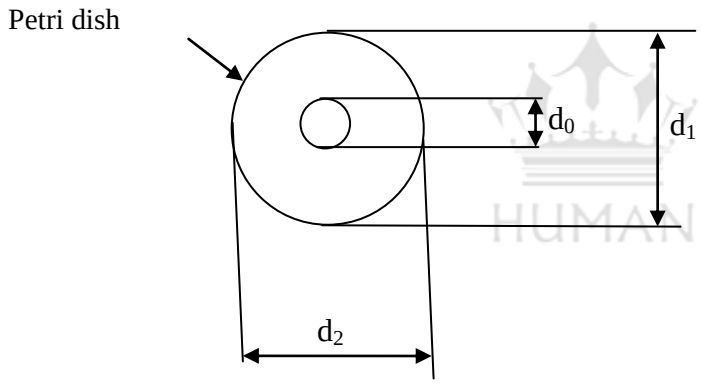
The radial growth of the pathogen was evaluated from the second day after inoculation, and every day until complete colonization of the control petri dish by the pathogen. This was done by measuring the two perpendicular diameters of the culture according to the formula of Singh and *al*., 1993.

$$D=\frac{(d1+d2)}{2}-d0$$

where:

D= radial growth

d_1 et d_2= perpendicular diameters of the culture



d_0= diameter of explant

Fig.2. Diagram illustrating the method of measurement of radial growth of du pathogen

Evaluation of fungicidal and fungistatic activities of different extracts

At the end of each test, the mycelium explants from the petri dishes in which growth was completely inhibited, were removed and aseptically deposited on the culture medium containing no extract. After 5 days depending on whether growth is resumed or not, the starting extract was identified as respectively fungistatic or fungicidal (Kishore and *al*., 1993, Pandey and *al*., 1982).

Correlation between concentration and percentage of inhibition

Correlation tests were performed in order to determine the relationship between the concentrations used and the percentages of inhibition obtained for each extract. In each cases, the correlation coefficient was determined in order to provide information on the degree of linear dependency; between the two variables. In this case, if a<0 then the relation is inversely proportional and the correlation is negative. If a >0 then the relation is positive; if r between 0.7 and 1 then the correlation is perfect and positive; if r is between -0.7 and -1 then the correlation is perfect and negative; if r is greater than -0.7 then the correlation is negative but imperfect.

Determination of minimal inhibitory concentrations

The values of the different percentages of inhibition made it possible to determine the minimum inhibitory concentrations. From the linear regression equation between the Neperian logarithms of the abscissa concentrations and the growth inhibition percentages on the y-axis, the concentrations reducing growth by 50% and 90% were determined (Dohou and *al*., 2004).

Statistical analysis

The data obtained were purified and subjected to statistical analysis using the xl-stat software which carries out the analysis of variances (ANOVA) for two factors. Duncan test was used to judge the differences between averages at 5% threshold.

RESULTS

Extraction yields

The extracts presented extraction yields according to the solvents used. For organic solvent, the yields were 39.02%; 38.04% and 26.02% respectively for acetone, ethyl acetate and methanol. The colour and appearance of the different extracts obtained depended on the different solvents used. The aqueous extract gave a yield of 30.04% (Table 1).

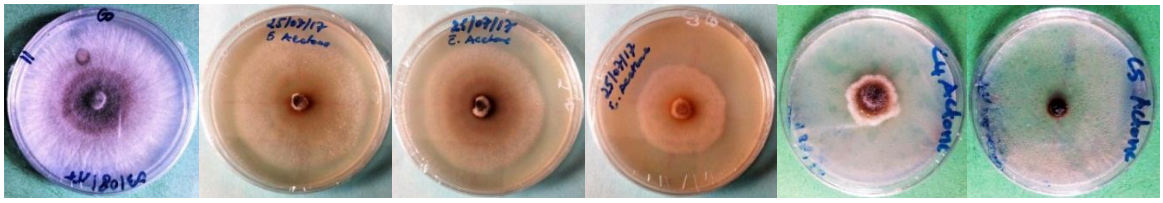
Table 1. Extraction yields and characteristics of extracts

Extraction solvent	Extraction yield (%)	Appearance	Colour
Acetone	39,02	Oily	yellowish
Ethyl acetate	38,04	Oily	yellowish
Methanol	26,02	Oily	yellowish
Aqueous	30,04	Oily	yellowish

Effects of different extracts on radial growth

Effects of acetone extract on radial growth

The acetone extract had an inhibitory effect on the radial growth of the strain. In view of the observations, the increase in concentration considerably inhibited the growth of the strain. A significant difference was revealed at 5% between the control and the different concentrations tested. Total inhibition growth was obtained with the C_5 concentration (120 µl / ml). At the fifth day, the C_1 dose (7.5 µl / ml) was similar in behavior to the control because no significant difference was observed (fig 3.)

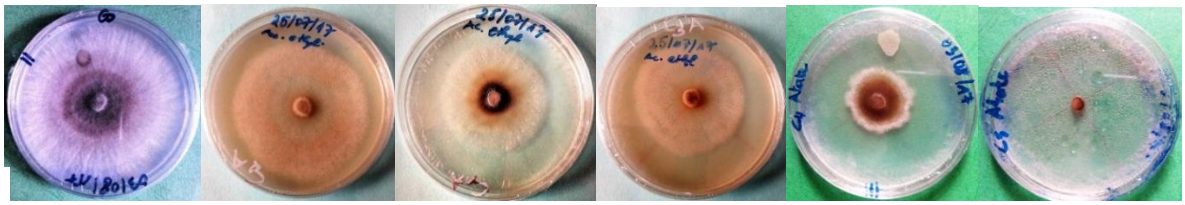


0 µl / ml 7,5 µl /ml 15 µl / ml 30 µl / ml 60 µl /ml 120 µl / ml

Fig. 3. Growth of strains in acetone extracts at different doses

Effect of ethyl acetate extract on radial growth

The ethyl acetate extract reduces the radial growth of the fungus. The diameter of the fungal colony decreased with increasing concentration towards zero at the highest concentration C_5 (120µl / ml) (Fig.4).

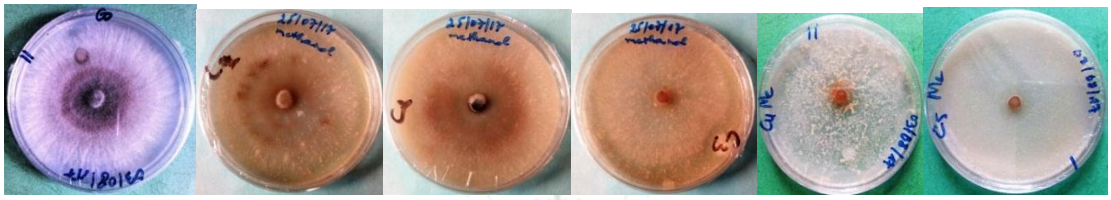


0 µl / ml 7,5 µl / ml 15 µl / ml 30 µl / ml 60 µl / ml 120 µl / ml

Fig. 4. Growth of strains in ethyl acetate extracts at different doses

Effect of methanol extract on radial growth

The methanol extract had a variable effect on the radial growth of the strain of *Cercospora malayensis*. The decrease or increase of the growth was not a function of the concentration because, at C_4, a radial growth greater than that observed at $C_2$was noticed. Complete inhibition of the fungal colony was obtained for the C_5 dose (Fig. 5.)

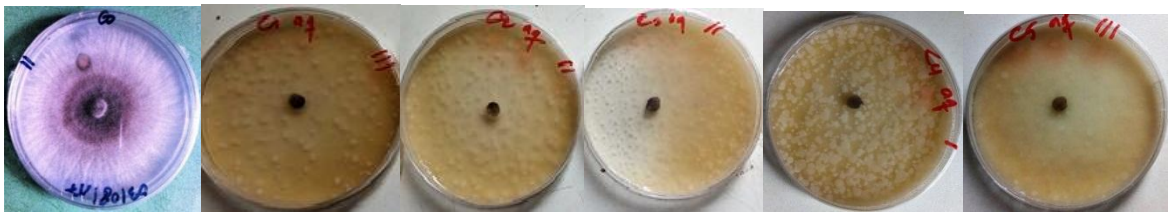


0 µl / ml 7,5 µl / ml 15 µl / ml 30 µl / ml 60 µl /ml 120 µl /ml

Fig. 5. Growth of strains in methanol at different concentrations

Effect of aqueous extract on radial growth

The aqueous extract was very effective in inhibiting radial growth. On the second day after seeding the strains, no mycelia growth was observed at different concentrations tested, unlike organic extracts. However, from the third to the fifth day, a low development was observed which immediately remained stable at 1.7 cm for C_1, $C_2$and C_3 doses (Fig. 6).



0 mg / ml 7,5 mg / ml 15 mg / ml 30 mg / ml 60 mg / ml 120 mg / ml

Fig. 6. Growth of strains in aqueous extract at different concentrations

Correlation test between concentrations and inhibition percentages

The purpose of this test was to see if there is a linear relationship between the decrease or increase in inhibition and the different concentrations of organic and aqueous extracts on the growth of the strain. The regression lines obtained after analysis revealed a similar behaviour of the strain with respect to the extracts (organic and aqueous). It appears that all the lines obtained have positive slopes and perfect correlations between the concentrations and the different percentages of inhibitions (Fig7).

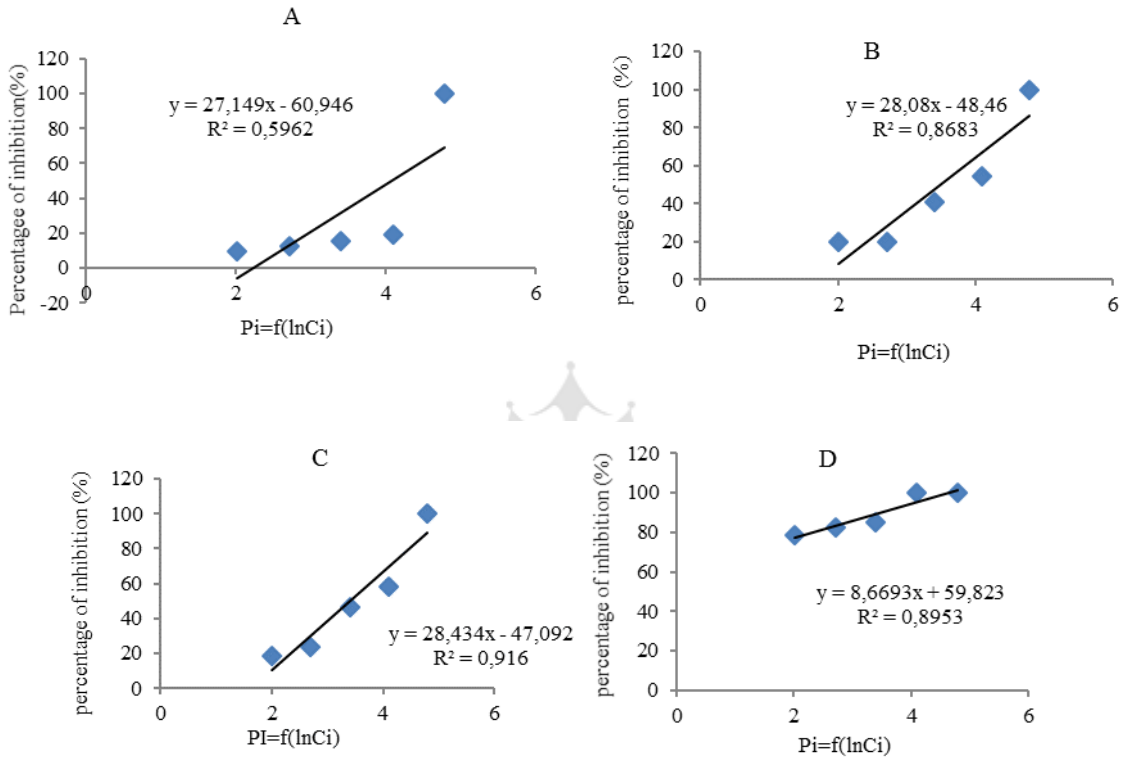


Fig.7. Regression lines for mycelia growth under different treatments (A= methanol extract, B= ethyl acetate extract, C= acetone extract, D= aqueous extract)

Minimal inhibitory concentration (MIC)

From the regression lines obtained after correlation tests, the 50% and 90% inhibitory concentrations (MIC_{50}, MIC_{90}) of the strain growth of the different extracts were determined. The lowest inhibitory concentrations were obtained with the different organic extracts. As for the aqueous extract, the MIC_{50} was indeterminate at zero statistically (Table II).

Table 2. MIC_{50} and MIC_{90} of mycelia growth of strain in different test extracts (µl/ml)

Extracts	MIC_{50}	MIC_{90}
Methanol extract	4,08	5,56
Ethyl acetate extract	3,50	4,93
Acetone extract	3,41	4,82
Aqueous extract	*	3,48

*Represent values that are not defined to be zero statistically

DISCUSSION

The objective of this work was to evaluate the fungicidal potential of aqueous and organic extracts of *Jartropha curcas* seeds on the radial growth of the strain of *Cercospora malayensis*. The sonicator extraction of 500 g of *J. curcas* seeds produced different yields according to the extracts. The lowest yield was obtained with the methanol extract (26.02). This variation in yield (39.02, 38.04, 20.02 and 30.04 respectively) for acetone, ethyl acetate, methanol and aqueous extracts could be attributed on one hand to the intrinsic factors of plant and to the organ considered on the other hand. Indeed, Svoboda and Hampson (1999) and Small field (2001) report that environmental conditions, harvest period and age of plant material could influence extraction yield. In addition, the polarity of the solvents could play a role in the extraction of many compounds (Mohammad and *al*., 2013).

The test carried out with the dilution solvent (alcohol 75 °) showed no significant difference between the control without solvent and the solvent-supplemented media. This test showed that the different surface-acting have any influence on radial growth of the fungus used. Similar results were obtained by Ambang and *al.*, 2010. Thus, the performances in the inhibition of the life stages of the fungus with *J. curcas* seed extracts obtained in this experiment testify to the fungicidal properties of these extracts.

The different extracts tested significantly reduced the growth diameter of the strain relative to the control. This reduction was more pronounced with the aqueous extract than with the organic extracts. These extracts (organic and aqueous) could contain substances that inhibit or retard the growth of the fungus. In fact, Domergue and Pirot (2008) underlined the presence of toxics protein in *Jatropha* seeds like curcine and lectine. In other hand, Makkar and *al*. (1997) shown the presence of some esterase, lipase and phorbol esters in the same

plant. Indeed, Lhost and *al*. (1993), Pamo and *al*. (2003) and Ling and *al*. (2003) reported that plant extracts from a number of plants contain compounds such as tannins, flavonoids and alkaloids that have fungicidal properties. The different concentrations of extracts tested significantly influenced the radial growth of the fungus with the highest concentrations being the most inhibitory. These results corroborate the work of Tih (2011) who obtained a reduction in the growth of *Colletotrichum gloeosporioides* strains by using acetone and methanol seeds extracts of *Thevetia peruviana*. Moreover, Zihiri and *al*. (2008) shown that the concentrations lower than 6 g/l of *Combretum racemosum* aqueous extract can totally inhibit the mycelia growth of *Pythium aphanidermatum*.

The inhibition percentages of plant extracts on the growth of *C. malayensis* strain also varied with increasing concentrations. At high concentrations, the organic and aqueous extracts exhibited total inhibition of fungus development such as that obtained with the use of metalaxyl + and mancozeb-based synthetic fungicides. In other words, the higher the concentration, the more the inhibition, which reveals the correlations observed between concentrations and percentage inhibition. Similar studies on antifungal activity of some extracts have been reported by Ngoh Dooh (2014ab); Doumbouya and *al.* (2012) and Ambang and *al*. (2010). These authors showed *in vitro* and *in vivo* efficiency of extracts of *Thevetia peruviana* on *Phytophthora megakarya*. On the other hand, the aqueous extract was more effective at low concentrations with more than 78% inhibition.

The MIC_{50} and MIC_{90} of various extracts were determined. The non-variability of low values of the MIC_{50} and MIC_{90} obtained demonstrated the efficiency and the fungicidal properties of different extracts of *J. curcas* on the growth of *C. malayensis* strains. These results agree with those of Doumbouya and *al*. (2012) who showed that the low MIC values highlighted the efficiency of an extract because they obtained a strong inhibition of the development of phytopathogenic fungi with *Ocimum gratissimum* extracts at low MICs.

CONCLUSION

The objective of this work was to evaluate the fungicidal potential of *Jatropha curcas* seed extracts on the strain of *Cercospora malayensis in vitro*. It appears that all the organic extracts tested inhibited the radial growth of the strain at the highest dose ((120 µl/ml). For the aqueous extract, the inhibition is obtained with the smallest dose (7.5 µl /ml). The inhibitory effect observed for the two types of extracts (aqueous and organic) is comparable to that of chemical fungicide used. In addition, the low MIC values obtained reveal the

effectiveness and highlight the fungicidal properties of these extracts. They contain a large number of secondary metabolites and can therefore be used as a pest control product in crop protection. These extracts were proven to be active on the strain of *Cercospora malayensis* and may be an alternative in the fight against fungal diseases of okra because their activity was comparable to that of synthetic fungicide (Monchamp).

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