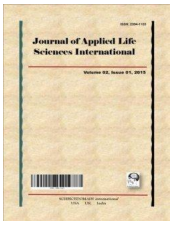

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Antifungal Potential of Aqueous Extracts of Neem Seeds (*Azadirachta indica*) on the *vitro* Development of Six Strains of *Phakopsora pachyrhizi*, Causal Agent of Asian Soybean Rust

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Authors' contributions

This work was carried out in collaboration between all authors. Authors NPA and AZ designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors ACC and NPA managed the analyses of the study. Authors MPED, HA and MSB managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

A study carried out in the University of Yaoundé I (Cameroon) aimed to evaluate the effect of aqueous extracts of neem seeds (*Azadirachta indica*) on the *in vitro* development of *Phakopsora pachyrhizi*. The experimental set-up was a completely randomized blocks design containing six treatments: T_0 (control), T_1, T_2, T_3 and T_4 (consisting of 12.5, 25, 50 and 100 µg/ml of extract respectively) and T_5 (5.33 µg/ml of Plantineb 80 WP). Six strains of *P. pachyrhizi* were used. The phytochemical screening of the aqueous extract of neem seeds (AENS), the effect of the extract on

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the fungi growth, the minimum inhibitory concentrations (CMI_{50} and CMI_{90}) and the characteristics of the extracts were determined by phytochemical and phytopathological methods. The results show that the AENS contains a varied range of chemical compounds such as sterols, sugars, alkaloids, saponins and terpenoids. The highest concentration of the tested extract (100 µg/ml) induced a total inhibition (100%) of the fungi growth. For all strains, the concentration of the extract was fungicidal. The inhibition percentage was proportional to the different doses of the tested extract and varies between 31.2 to 71.4% depending on the strains. MIC 90 was 81.8, 82.8, 83.7, 82.9, 84.4 and 84.8% for the F.Mi, F.Nko, G.Mi, G.Nko, Gr.Mi and Gr.Nko strains respectively. Thus from this study, the efficacy of high doses of AENS is similar to that of Plantineb 80 WP in the *in vitro* control against *P. pachyrhizi*. This substance could thus be included in the control program for the pathogen.

Keywords: Antifungal activity; Phakopsora pachyrhizi; aqueous extracts; Azadirachta indica.

1. INTRODUCTION

Soybean (*Glycine max*), an annual plant of the Fabaceae family, is present in more than 80 countries worldwide. Native from East Asia [1], it is grown mainly for its highly rich seeds in protein (40%), fat (20.2%) and trace elements [2]. The world production of soybean increased considerably during the 20th century. FAO currently estimates it at approximately 315 million tons; the main producers are the United States, Brazilia and Argentina with about 82%. However this very high productivity results from the increase of cultivated surfaces inducing large deforestation. In South America, cultivated surfaces passed from 18 to more than 40 million hectares [3]. Today, in Cameroon and many other African countries, soybeans are widely used in various forms of food to curb malnutrition. However, multiple infections substantially reduced the productivity of soybeans. Several fungal diseases affect many varieties of soybean in the field; besides, the Asian rust is nowadays the most widespread disease in the world. Two main methods of control are usually used to remedy these pathologies, namely genetic and chemical control. These individual methods have shortcomings and are constraining to the environment [4]. Chemical control is the most used and most effective; based primarily on the use of synthetic pesticides it is costly and harmful because of the concentration of residues in food chains [5]. In addition, improved varieties used in genetic control do not unfortunately totally resist against these diseases. An alternative, the use of environmentally friendly biopesticides is conducive to healthy and sustainable agriculture. In the search of natural pesticides, neem (*Azadirachta indica*) is one of the most widely used plant. This plant of the Meliaceae family is originally from Eastern India. The pesticide potential of neem tree has already been demonstrated in the control of several crop parasites. Thus, it has fungicidal [6], insecticide [7,8], and nematicide [9] properties. Its effectiveness in the control of the Asian rust of soybean would enhance the protection of plants in the field and the environment.

2. MATERIALS AND METHODS

2.1 Materials

Six *P. pachyrhizi* strains collected on leaves (F), pots (G) and grains (Gr) in the two sites of the study, Mimetala (Mi) and Nkometou (Nko) were used. According to these abbreviations, the six strains were named: F.Mi, F.Nko, G.Mi, G.Nko, Gr.Mi and Gr.Nko. The plant material consisted of neem seeds and the chemical material used was Plantineb 80 WP which contains 80% of *maneb* (Fig. 1). Several other laboratory materials were used.

2.2 Methods

2.2.1 Preparation of aqueous extracts of neem seeds

Mature fruits harvested in Maroua (Cameroon), were used to collect the seeds. These were then dried at ambient temperature for 2 to 3 days and crushed. A stock solution of 0.5 g/ml concentration was prepared by introducing 50 g of neem seeds into 100 ml of distilled water and kept for about 12 hours, and filtered with the muslin tissue. Using the formula CiVi = CfVf [10], four concentrations were prepared from the stock solution: 0.0125, 0.025, 0.050 and 0.1g/ml by adding 1.5, 3, 6 and 12 ml of the stock solution to 58.5, 57, 54 and 48 ml respectively of PDA to make 60 ml.

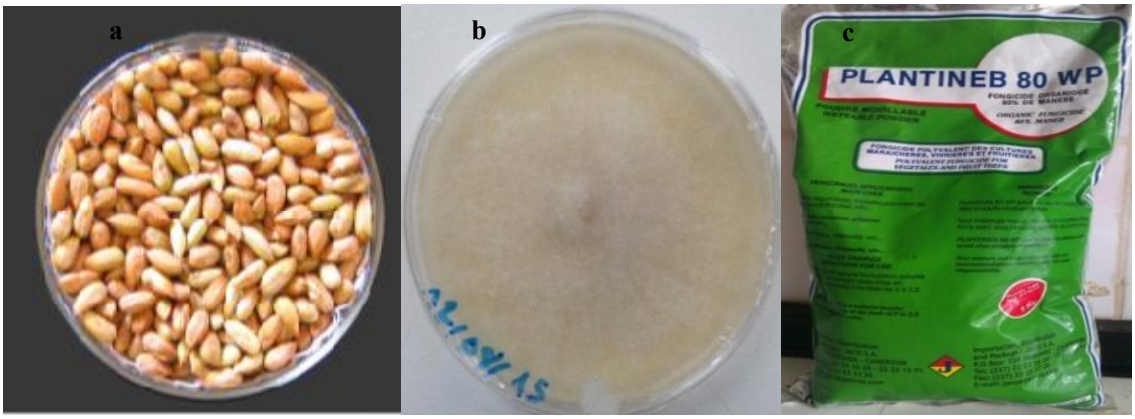

Fig. 1. Main materials used (a: neem seeds, b: Pure strain of *P. pachyrhizi* (Gr.Nko), c: chemical fungicide)

2.2.2 Phytochemical screening of aqueous extracts of neem seeds

Classes of secondary metabolites in aqueous extracts of neem seeds were determined by adapting standard procedures according to [11]. These techniques are based on the turbidity, precipitation and foam of the extracts in the presence of the different reagents characterizing each class of secondary metabolites. A small volume of the extract was used to determine the qualitative presence of alkaloids, phenols, triterpenes, sterols, flavonoids, saponins, anthocyanin, glycosides and tannins. A volume of 0.5 ml of extract was especially added to 1ml of sulfuric acid (H_2SO) in test tubes, and then homogenized for 2 min. After boiling, 5 drops of Meyer's reagent was added into each tube; the turbidity noted indicates the presence of alkaloids. About 0.5 ml of each extract was mixed in 1 ml of methanol in a tube bearing the extract, and then heated in a bath at 55°C for 15 min. Then, after adding three drops of freshly prepared ferric cyanide to the mixture, a green precipitate occurred indicating the presence of phenols. After that, 0.5 ml of each extract was added in the test tubes containing HCl previously neutralized by 5% sodium hydroxide (NaOH). And the apparition of a brick red precipitate is the indication of the presence of glycosides. To evaluate saponins, terpenoids, sterols, tannins, anthocyanins and flavoids, adapted techniques of [12] were used.

2.2.3 Isolation and purification of strains

Organ samples (leaves, pods and seeds) of soybeans showing the symptoms of the disease were identified and harvested preferably in the most affected areas of the plantation. After bagging and labeling, the organs were taken to the laboratory; there they were washed thoroughly several times with tap water. The leaves and pods were cut into sections of about 1 cm^2 each, while the diseased seeds were used in their entirety. After disinfecting them with 95° alcohol for 2 min, they were rinsed twice with sterile distilled water [13]. With the scalpel, sections of aseptic organs were placed in Petri dishes containing the PDA. The incubation took place at a temperature of 25°C with a photoperiod of 12 h. Five (05) days later, necrosis fragments were removed and transferred to new Petri dishes containing the PDA. This operation was repeated several times in order to obtain pure strains (Fig. 1 b).

2.2.4 Effects of the EAGN on the fungal strains development

2.2.4.1 Radial growth

A necrosis fragment (diameter = 0.6 cm) was placed in the center of each Petri dish containing the different treatments (T0, T1, T2, T3, T4, consisting of: 0.0125, 0.025, 0.05 and 0.1 g/ml of extract respectively, and T5 constituted 5.33 µg/ml of plantineb 80 WP). Each treatment was repeated three times. The diameter of the necrosis was measured every day from the 2nd day after seeding, on perpendicular lines passing through the center of the explants (Fig. 2). The radial growth average (D) is determined using the formulae:

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

Where: d_0 is the diameter of the initial explant; d1 and d2 are the culture diameters measured in two perpendicular directions.

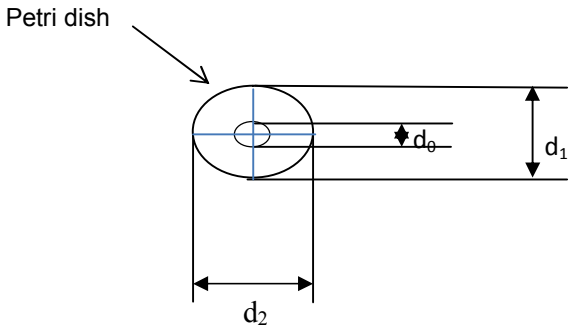

Fig. 2. Principle for measuring mycelial growth in Petri dishes

2.2.4.2 Aggressiveness and susceptibility of the fungal strains

The aggressiveness of strains is determined by the growth rate of the pathogen in Petri dishes containing only the PDA. Indeed, the radial growth of the different strains is measured every day until the maximum growth of one of them, and then the average dimensions are determined and compared between them. The strain with the largest diameter is said to be the most aggressive and if not, the least aggressive.

The sensitivity of a strain to the extract is function of the percentage of inhibition. The strain having the highest percentage of inhibition is said to be very sensitive to the extract whereas that with the lowest percentage of inhibition is considered as the least sensitive or the most tolerant. The percentage of inhibition of the aqueous extracts of neem seeds is determined in the various treatments adapting the following formula:

$$I(\%) = \frac{Dt-Dx}{Dt} x\ 100$$ [14]

With: I (%) = percentage inhibition; Dt = Average diameter without neem extracts, Dx = Average diameter with neem extracts.

2.2.5 Fungicidal or fungistatic activities of extracts

After incubation, the explants resulting from the treatments induced the complete inhibition of the growth of the fungi, were transferred to an environment free from extracts. It is noted that if the growth of the fungus is interrupted several days after seeding in the new milieu, the extract is said to be fungicidal; if it resumes, it is instead fungistatic [15,16].

2.2.6 Determination of Minimum Inhibitory Concentrations

The values of the various inhibition percentages permitted to determine the minimum inhibitory concentrations reducing 90% of the mycelial growth (MIC 90), through the linear regression equation.

2.2.7 Correlations between concentration and inhibition

The correlation between concentration and inhibition was determined from the equation $y = ax + b$ with a = slope, b = constant, x = logarithm of the concentration, and y = percentage inhibition and r the correlation coefficient. In this case, if $a < 0$, the slope is negative; if $a > 0$, it is positive; if $0.8 < r < 1$, the correlation is perfect and positive (the inhibition is strictly proportional to the concentration); If $-0.8 < r < -1$, the correlation is perfect and negative (the inhibition is inversely proportional to the concentration); if $r < 0.8$ then the correlation is positive but imperfect; if $r > -0.8$ then the correlation is negative but imperfect [17].

2.3 Statistical Analysis

Data obtained for all the studied parameters were subjected to an analysis using the software S.A.S version 9.0; Different averages were compared using ANOVA with the probability threshold p = 0.05. Histograms were plotted using Microsoft Office Excel 2010 software.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Phytochemical screening of aqueous extracts

Phytochemical screening of the neem seeds extracts revealed various chemical compounds belonging to different families (alkaloids, saponins, terpenoids, sterols and sugars). Tannins, glycosides, anthocyanins, flavonoids, glycosides and phenols were absent (Table 1).

Table 1. Natural products in the extracts: - absent, + present, +++ abundant

Class of secondary metabolites	Presence in extract
Alkaloids	+
Saponins	+
Terpenoids	+
Sterols	+++
Tannins	_
Phenols	_
Anthocyanins	_
Sugars	+++
Flavonoids	_

3.1.2 Effect of the aqueous extract of neem seeds (AENS) on the development of strains

The aqueous extract of neem seeds significantly reduced the mycelial growth of all strains used and at all doses tested. For the first three doses tested (0.0125, 0.025 and 0.050 g/ml), the inhibition percentage varied from 31.2 to 71.4%. The lowest value was recorded on the G.Nko strain at the dose of 0.0125 µg/ml and the largest on the F.Mi strain at a dose of 0.05 g/ml. The highest extract dose (T4 = 0.1 g/ml) on all the strains gave a total inhibition (100%). The effect of this dose is similar to the systemic and synthetic fungicide used (Fig. 3).

3.1.3 Aggressiveness and susceptibility of strains

3.1.3.1 Aggressiveness of strains

The development of *P. pachyrhizi* strains observed in the control treatments shows a very similar level of aggressiveness. From the second day after incubation, the radial growth on F.Mi and F. Nko strains was 1.5 against 1.8 for the four others. In addition, 6 days after incubation, G.Mi, G.Nko, Gr.Mi and Gr.Nko strains showed higher radial diameters than the two others (7.7 versus 7.1 cm respectively), showing a fairly high level of aggressiveness (Table 2).

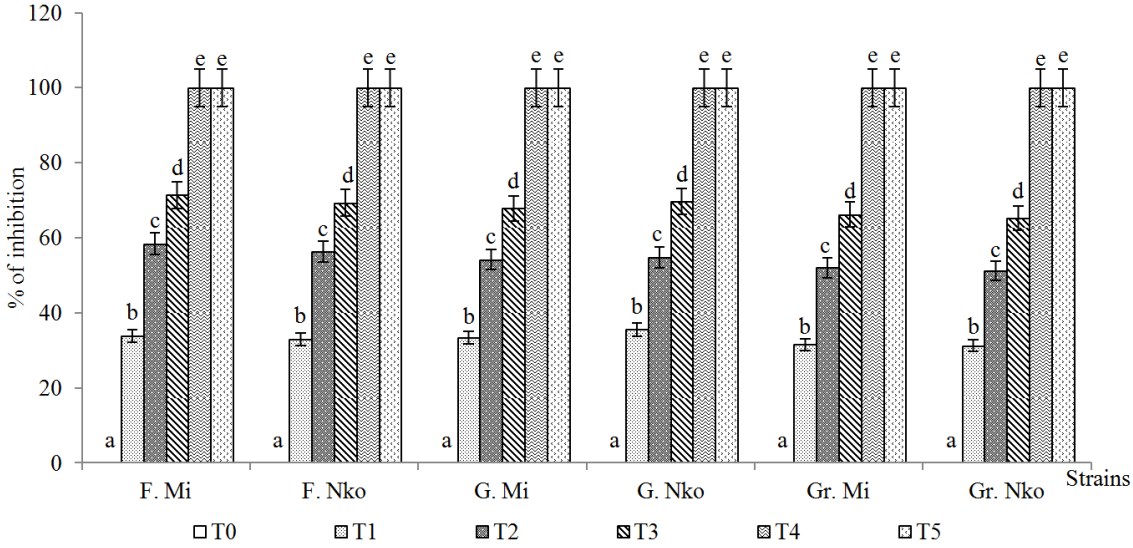

Fig. 3. Effect of AENS on the growth of different strains of *P. pachyrhizi*.
** Values of the same strain with different letters are significantly different at p <0.05.*

Table 2. Radial growth of different strains as a function of time

	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
F. Mi	1,4	2,75	4,2	6	7,1	7,9
F. Nko	1,5	2,9	4,4	6,1	7,2	7,9
G. Mi	1,7	2,95	4,45	6,2	7,5	7,9
G. Nko	1,8	3	4,6	6,4	7,7	7,9
Gr. Mi	1,7	2,95	4,6	6,3	7,6	7,9
Gr. Nko	1,8	3	4,6	6,4	7,7	7,9

3.1.3.2 Sensitivity of different strains to the extract

The level of sensitivity of the tested strains is different with respect to the different concentrations of the extracts applied. It is noted that for all strains, the highest concentration of the extract of neem seeds (0.1 g/ml) totally inhibited mycelial growth. The monitored Gr.Mi and Gr.Nko strains and G.Mi and G.Nko strains showed the lowest inhibition percentages at all doses and thus provinga tolerance to neem seed extracts. However, the F.Mi and F.Nko strains showing the highest inhibition percentages at all concentrations tested are more sensitive to AENS (Table 3).

Table 3. Inhibition (%) of the six strains in the different treatments

	T1	T2	T3	T4
F. Mi	33,9 b	58,4 a	71,4 a	100,0 a
F. Nko	33,0 b	56,3 b	69,3 b	100,0 a
G. Mi	33,5 b	54,2bc	67,8 d	100,0 a
G. Nko	35,5 a	54,8 c	69,7 c	100,0 a
Gr. Mi	31,6 c	52,0 d	66,2 e	100,0 a
Gr. Nko	31,2 c	51,3 d	65,3 f	100,0 a

F.Mi= strains collected on leaves in Mimetala, G: on pots, Gr: on grains, Nko: in Nkometou

3.1.4 Fungicidal and fungistatic activity of extracts and plantineb 80 WP

The synthetic fungicide (plantineb 80 WP) showed a higher inhibitory effect on all the six strains. Indeed, it is proved to be fungicidal. The first three concentrations tested (0.0125, 0.025 and 0.050 g/ml aqueous extract of neem seeds) exhibited fungistatic activity for all strains. However, the highest extract dose (0.1 g/ml) was revealed fungicidal on all strains (Table 4).

3.1.5 Variation of the minimum inhibitory concentration (MIC90)

The different regression lines of different strains helped to determine the minimum inhibitory concentration. The F.Mi strain shows the lowest minimal inhibitory concentration of the extract while the Gr. Nko strain shows the highest minimum inhibitory concentration Table 5).

3.1.6 Correlation between concentration and inhibition percentage

The regression lines of all strains show positive slopes. Moreover, their regression coefficients (r) are high, above 0.8 and close to 1(0.97 and 0.98). These values indicate a high correlation between concentration and inhibition percentage. In other words, the inhibition is strictly proportional to the concentration of the extract used.

Table 4. Fungicidal and fungistatic activity of extracts on *P. pachyrhizi* strains

Strains	Concentrations	Effect
F. Mi	C1	Fungistatic
	C2	Fungistatic
	C3	Fungistatic
	C4	Fungicide
F. Nko	C1	Fungistatic
	C2	Fungistatic
	C3	Fungistatic
	C4	Fungicide
G.Mi	C1	Fungistatic
	C2	Fungistatic
	C3	Fungistatic
	C4	Fungicide
G.Nko	C1	Fungistatic
	C2	Fungistatic
	C3	Fungistatic
	C4	Fungicide
Gr.Mi	C1	Fungistatic
	C2	Fungistatic
	C3	Fungistatic
	C4	Fungicide
Gr.Nko	C1	Fungistatic
	C2	Fungistatic
	C3	Fungistatic
	C4	Fungicide

C1=0.0125, C2=0.025, C3=0.050 and C4=0.1 g/ml.

3.2 Discussion

The phytochemical screening of aqueous extracts of neem seeds revealed the presence of several chemical compounds of different families such as saponins, terpenoids, sterols and alkaloids. These results show that plants with a pesticidal potential generally contain these secondary metabolite categories as demonstrated by [18] and [19] respectively on aqueous extracts of *Thevetia peruviana* and *Azadirachta indica*.

Table 5. Concentrations reducing 90 % (MIC_{90}) of the mycelial growth of strains

Strains	F. Mi	F. Nko	G. Mi	G. Nko	Gr. Mi	Gr. Nko
MIC_{90} (µg/ml)	81,8	82,8	83.7	82,9	84,4	84,8

The aqueous extracts at the three concentrations used showed satisfactory percentages of inhibition ≤ 71.4%). At the concentrations C1, C2 and C3, the increasing average of inhibition observed show that the efficacy of the product is proportional to its concentration. These results are similar to those obtained by [20] using aqueous extracts of *A. indica* leaves on the mycelial growth of *Sclerotium rolfsii* strains. There was a total inhibition of all strains with the highest concentration (0.1 g/ml). The sensibility of the strains at this concentration of neem extract can be due to the specificity of its cell composition and structure as that was shown in similar results obtained by [21] with essential oils of *Hyptis marrubioides* on the germination of *P. pachyrhizi* spores. These antifungal properties of the aqueous extract of *A. indica* seeds can be explained by the presence of sterols, essential oils (Terpenoids) and sugars which are secondary metabolites with very strong antifungal potential. At a high dose (0.1 g/ml for example), the extract appeared fungicidal, as in the study of [22] where this same extract presented a similar effect on some *Phytophthora megakarya* strains at the dose of 50 µg/ml. Thus, at high doses, this extract could be used to fight against *P. pachyrhizi*.

Table 6. Correlation between inhibition percentage of strains and concentrations of AENS

Different strains	Correlation coefficients (r)	Observations
F. Mi	0,97	Highly correlated
F. Nko	0,97	Highly correlated
G. Mi	0,98	Highly correlated
G. Nko	0,98	Highly correlated
Gr. Mi	0,98	Highly correlated
Gr. Nko	0,98	Highly correlated

Correlation tests carried out were designed to establish linear relationships between the concentrations of aqueous extracts tested and the inhibition percentages of each strain used. The correlation coefficients determined (r = 0.97 and 0.98), showed that these parameters are highly correlated, allowing to understand the degree of dependence existing between them. In reality, the extract concentration increases with the inhibition percentage. It means that, the inhibition percentage is strictly proportional to the different concentrations of the used extracts. This phenomenon observed for the case of neem seed extracts in this work shows that there was no antagonism or blockage in the increase of the extract doses on the fungus growth inhibition process. These results confirm those obtained by [23] in similar experiments on powder extract and essential oil of *Xylopia aethiopica* against tomato *Fusarium oxysporum*.

4. CONCLUSION

In this study, it has been demonstrated that the aqueous extracts of neem seeds reduced significantly the mycelial growth of all the *P. pachyrhizi* strains used. The inhibitory effect of each extract was proportional at various doses tested. At a high dose (0.1 g/ml) the extract showed fungicidal activity, similar to that of plantineb 80 WP, the synthetic fungicide. Thus, this extract could be used as an alternative to the chemical fungicide.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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