Archives of Phytopathology and Plant Protection
Vol. 43, No. 10, 1 July 2010, 931–939

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In Vitro toxicity of oil extracted from neem seeds collected from different locations across savanna agro-ecological zones of Nigeria on seed and soil-borne pathogens of cowpea (*Vigna unguiculata* L. Walp)

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(*Received 24 February 2008; final version received 19 March 2008*)

Graded extracts of neem seed oil extracted across the savanna agro-ecological zones (AEZs) of Nigeria were tested on mycelial growth of *Colletotrichum capsici* and *Macrophomina phaseolina* of cowpea in Nigeria. Minimum inhibition concentrations (MIC) and effectiveness levels of oil from AEZs were determined. Data were subject to ANOVA. Biplot was employed to access variation and interactions among the AEZs. Across the AEZs, fungitoxicity, MIC and effectiveness level varies; concentrations of 0.1 and 1.0% reduced the growth of *C. capsici*, while *M. phaseolina* was reduced at 10% concentration. On *M. phaseolina*, neem oil extracted from Ilorin, Ogbomosho (derived savanna) and Mokwa (southern guinea savanna) performed similarly, while a strong positive association existed between Ogbomosho (derived savanna) and Hadejia (Sahel savanna) samples. On *C. capsici*, samples from Bida (southern guinea savanna) and Ilorin (Derived savanna) were similar. This result could be a possible link between ecology, biodiversity and toxic principles in plant materials.

Keywords: savanna agro-ecological zones; neem seed oil; cowpea pathogens; concentrations

Introduction

Cowpea (*Vigna unguiculata* (*L.*) Walp.) is an indigenous food legume in the dry savanna of Africa, especially West Africa. Although it is cultivated worldwide, over 75% of the world production occurs in Africa with Nigeria and Niger constituting the world's largest producers (Coulibaly and Lowenberg-DeBoer 2002). It is variously used as food grain, vegetables, animal feed as well as a source of income for the poor sectors. Cowpea grain is very high in protein and vital minerals. Cowpea is valuable as cover crop as well as providing opportunities for earning cash, particularly among women. Trading from fodder has been estimated to increase income by as much as 25% of their normal annual income while fodder yields of up to 0.5 t/ha are commonly obtained in northern Nigeria (Quin 1997; Fatokun et al. 2002). West Africa accounted for about 92% of 10.4 million ha worldwide of cowpea and produced 84% of the 3.6 million ton of dry grain in 2005 (FAO 2006; Emechebe and Florini 1997; Florini 1997; Emechebe and Lagoke 2002). A range of diseases attacks cowpea across agro-ecological zones of West Africa. Almost all parts of

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ISSN 0323-5408 print/ISSN 1477-2906 online

DOI: 10.1080/03235400802144413
http://www.informaworld.com

the plant are affected (Singh and Allen 1979; Emechebe and Florini 1997). Fungal diseases of cowpea are numerous and often have devastating effects. Almost 40 fungal species are known to be pathogenic to cowpea (Allen 1983), their occurrence varying according to different agro-ecological zones. Important cowpea diseases in the moist savanna include seed decay and damping-off caused by *Macrophomina phaseolina* (Tassi) Goid, *Pythium* spp, and anthracnose (*Colletotrichum destructivum* O Gara) and brown blotch (*Colletotrichum capsici*, (Syd.) Butler and Bisby) and bacterial blight, (*Xanthomonas axonopodis* pv *vignicola*) (Burkholder) Dye and Sphaceloma scab (*Elsinoe phaseoli* Jenkins) (Emechebe and Shoyinka 1985). Estimates of crop losses due to these diseases have been reported by various researchers. For example, under favourable conditions, *Macrophomina* blight caused up to 68% crop losses in Niger (Afouda 1999) while brown blotch caused up to 46% yield loss in Nigeria northern guinea savanna (Alabi 1994). Often, the use of synthetic fungicides are a very effective control option (Alabi and Emechebe 1992). However, their long-term effects on health, the natural environment, risk of pathogen resistance to fungicide usage, and the quality of agricultural products are of serious concern to all (Coulibaly and Lowenberg-DeBoar 2002).

Biopesticide control options are suggested alternatives; they are attractive due to their potential of being environmentally friendly. Some workers however, consider that they are often low in efficacy and are therefore not effective (Ewete et al. 1996). However, some highly effective natural plant products with potential for use as pest and disease control agents exist. Neem is an example of a botanical with such uses. It is of tropical origin and popularly known for its risk-free effect to beneficial microorganisms. The safety of neem derivatives to beneficial microorganism, rapid biodegradability and environmentally friendly properties make it an ideal pesticide (Dales 1996; Onalo 1999). Neem nativity has been traced down to the arid and semi-arid India, hence its inherent ability to survive maximally in the dry savanna agro-ecologies. Savanna and semi-arid agro-ecologies of Nigeria are divided into five major zones. These are the derived savanna, southern guinea savanna, northern guinea savanna, Sudan savanna and the Sahel, occupying about 80% of Nigerian land. Over 70% of West African land is situated in the savanna zones (Isichei and Awodoyin 1990; Awodoyin et al. 1997). Although many workers have reported their findings on pesticidal properties of neem (Saxena 1990; Ivbijaro 1993, 1990; Ketkar and Ketkar 1993; Lale and Abdulrahaman 1999; Onalo 1999), little attention has been paid to the diversity of neem across the savanna agro-ecological zones of Nigeria. Therefore, the objective of the present study was to evaluate the variation in the effectiveness of neem seed oil from neem grown across the savanna AEZs against *M. phaseolina* and *C. capsici*.

Materials and methods

Field AEZs survey

Neem fruits were collected from around 10 cities and towns across the savanna agro-ecological zones (AEZs) of Nigeria. Collection sites were spread over the distinct agro-ecological zones at about 20 km intervals within an AEZ. The collection was done between September 5 and 12, 2002. The location at which samples were collected is shown in Table 1.

Sampling procedure employed in collecting neem seed across the AEZs

Sampling was done based on trunk diameter, tree height and the agro-ecological zone. About 20–25 mature trees were sampled per zone. Selection of trees to be

Table 1. Places from which neem seeds were collected in Nigeria.

Agro-ecological zones of collection sites	State	Location of collection site
Derived Savanna	Oyo	Ogbomosho (8°13'N,4°26'E)
	Kwara	Ilorin (8°50'N,4°55'E)
Southern Guinea Savanna	Niger	Mokwa (9°18'N,7°38'E)
	Niger	Bida (9°05'N,6°01'E)
Northern Guinea Savanna	Kaduna	Kaduna (10°52'N,7°44'E)
	Kaduna	Zaria (11°11'N,7°38'E)
Sahel Savanna	Jigawa	Hadejia (12°45'N,10°05'E)

sampled was restricted to those that were fruiting and had a trunk diameter of 30 cm or more at 1 m above the soil level. Approximately 3 kg of dropped ripened yellow fruits were picked under neem trees that were sampled. The fruits were placed in a paper bag, properly labelled and returned to the IITA screen house at Ibadan, Nigeria for air-drying.

Preparation of neem seed oil

The extraction was carried out using analytical grade hexane; 100 g of the pulverized neem seed was weighed with Mettler PI65 balance and wrapped in a double layer of Whatman^{R} No.1 filter paper. The paper was sealed up at edges to prevent spillage. Each pack was then extracted in 200 ml hexane for 6 hrs at 70°C. The weight of oil extracted was expressed as a percentage of the weight of neem seed powder used for the extraction as described by Lale and Abdulrahman (1998).

Bioassay technique employed during the study

All *in vitro* studies to test the efficacy of the neem seed oil were done on acidified potato dextrose agar APDA; the extract was acidified to pH of 5.7 using lactic acid. Stock solution of the neem seed oil was prepared by measuring 10 ml each of the extracted neem seed oil from different location and added to 90 ml molten APDA. The neem seed oil was tested at concentration of 0.01, 0.1, 1.0, and 10.0%. Mycelial agar plugs were taken with cork borer no 1 (diameter 3 cm) from the margin of one week-old culture of test pathogens, namely, *C. capsici* and *M. phaseolina*. These were placed at the centre of each Petri dish containing the seed oil in molten APDA, and the plates were then incubated at 28–30°C for seven days. Sterile distilled water applied to molten APDA instead of neem seed oil served as control. In particular, radial growth of each fungus was measured as the mean growth along the two axes on two pre-drawn perpendicular lines on the reverse side of the plate.

The percentage fungitoxicity was expressed as described by Awuah (1989) as:

$$MP = \frac{M1 - M2}{M1} \times 100$$

where MP = percentage inhibition of mycelial growth, M1 = mycelial radial growth in control Petri dish without extract, and M2 = mycelial radial growth in Petri dish containing extract

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) as described by Ejechi and Souzey (1999) is the lowest concentration of the extract that prevents fungal growth. This was determined to guide the selection of effective concentrations of neem seed oil and the most suitable AEZ for the production of fungitoxic neem seed oil. Neem seed oil extracts were rated based on their inhibitory effects using a modified scale as described by Sangoyomi (2004):

– 0% = stimulatory (ST),
0% inhibition = no effect (NE),
1–20% inhibition = slightly effective (SE),
21–40% inhibition = moderately effective (ME),
41–60% inhibition = effective (EE), and
61% inhibition and above = highly effective (HE).

Statistical analyses

All experiments were repeated twice. Percentage growth inhibitions were subject to analysis of variance (ANOVA) using the generalised linear model procedure (GLM) of statistical analysis software (SAS Institute version 8, 2001). Means comparison was effected at $P \leq 0.05$. Biplot analysis was employed to access the variation and possible similarities within the locations studied.

Results

MIC and efficacy of neem extracted from seeds collected from different locations

Determination of the minimum inhibition concentration (MIC) of oil from various locations and AEZs on *M. phaseolina* showed that 1.0% and 10.0% concentrations of neem seed extract in APDA were effective for reducing the mycelial radial growth of *M. phaseolina* (Table 2). Moderately effective (ME) levels were observed in 10% neem seed oil extracted from seeds collected from Ogbomosho (in derived savanna) AEZ Hadejia (in the Sahel savanna), AEZ and Zaria (in northern Guinea savanna) AEZ (Table 2). With the exception of oil from seeds collected from Mokwa (southern Guinea savanna), neem seed oil extract obtained from seeds collected from other locations were either highly effective (HE) or effective (EE) on *C. capsici* at 10% concentration (Table 3). It is noted that toxicity to *C. capsici* occurred even at 0.01 and 0.1% concentrations of the neem seed oil regardless of the location of the seeds from which the oil was extracted. In general for each of the pathogens, the *in vitro* reductions in radial mycelia growth caused by neem seed oil extracted from various locations are concentration-dependent and significantly ($P \leq 0.05$) different from one another (Table 2 and 3).

AEZ variations and interactions

Interaction and variations across the AEZs and among the concentrations tested on neem seed oil were evaluated. Biplot analysis serves to access relations among entries and environments and between entries and environments. Entries close to the origin possess similar effects. A small angle between two environmental vectors indicates a strong positive association (similarities) between the two environments. An angle of 90^0 indicates

Table 2. Effect of site of collection of neem seed, and concentrations of neem seed oil on radial growth of *Macrophomina phaseolina* in acidified PDA.

Locations of neem seed collection	Radial mycelial growth inhibition % in APDA at neem seed oil concentration of[a]					
	0.01	0.1	1.0	10.0	Minimum effective concentration[c]	Level of Effectiveness[b]
Ogbomosho	0	0	3.7	25.9	1.0	(ME)
Ilorin	0	0	0	0	NA	(NE)
Mokwa	0	0	0	0	NA	(NE)
Bida	0	0	0	0	NA	(NE)
Kaduna	0	0	0	0	NA	(NE)
Zaria	0	0	0	24.1	10.0	(ME)
Hadejia	0	0	19.6	33.3	1.0	(ME)
Overall mean	0	0	3.33	11.9		
SE±	0	0	3.24	5.70		

[a]Values are means of three replicate plates per treatment; [b]NE = not effective; ME = Moderately effective; NA = not applicable, [c]MIC = minimum inhibition concentration.

Table 3. Effect of site of collection of neem seed and concentrations of neem seed oil on radial growth of *Colletotrichum capsici* in acidified PDA.

Locations of neem seed collection	Radial mycelial growth inhibition % in APDA at neem seed oil concentration of[a]					Level of Effectiveness[b]
	0.01	0.1	1.0	10.0	MIC[c]	
Ogbomosho	3.9	36.2	36.7	66.1	0.01	(HE)
Ilorin	15.2	21.6	37.3	43.5	0.01	(EE)
Mokwa	23.7	23.7	28.2	37.3	0.01	(ME)
Bida	16.7	23.2	37.9	44.1	0.01	(EE)
Kaduna	20.5	35.6	37.9	65.3	0.01	(HE)
Zaria	ND	45.8	55.4	54.8	ND	(EE)
Hadejia	23.8	35.0	50.3	74.6	0.01	(HE)
Overall mean	17.3	31.59	40.53	55.1		
SE±	3.22	3.39	3.47	5.29		

[a]Values are means of three replicate plates per treatment; Effectiveness[b]; NE = not effective; ME = Moderately effective; EE = Effective; HE = Highly effective; [c]MIC = minimum inhibition concentration.

no association while an angle greater than 90^0 represent dissimilarities (Hess et al. 2002). Taking this into consideration the results indicate that within the locations, the effect of neem seed oil on *M. phaseolina* showed that Ilorin and Ogbomosho both in derived savanna and Mokwa in southern Guinea savanna had similar effects. Hadejia in Sahel savanna produced an average effect while a strong positive association existed between Ogbomosho Derived savanna and Hadejia Sahel savanna and these were supported by Table 2 and Figure 1. In the case of *C. capsici*, oil extracts from Bida southern Guinea savanna and Ilorin in derived savanna (DS) had similar effects, a conclusion borne out by Table 3. Kaduna northern guinea savanna sample gave an average effect and a positive association existed between Kaduna and Hadejia samples (Figure 2). The results showed that a similar performance was obtained at neem seed oil concentrations of 0.01%, 0.1% and 1.0% as they all performed averagely, while 10.0 and 0% are not similar (Figure 3).

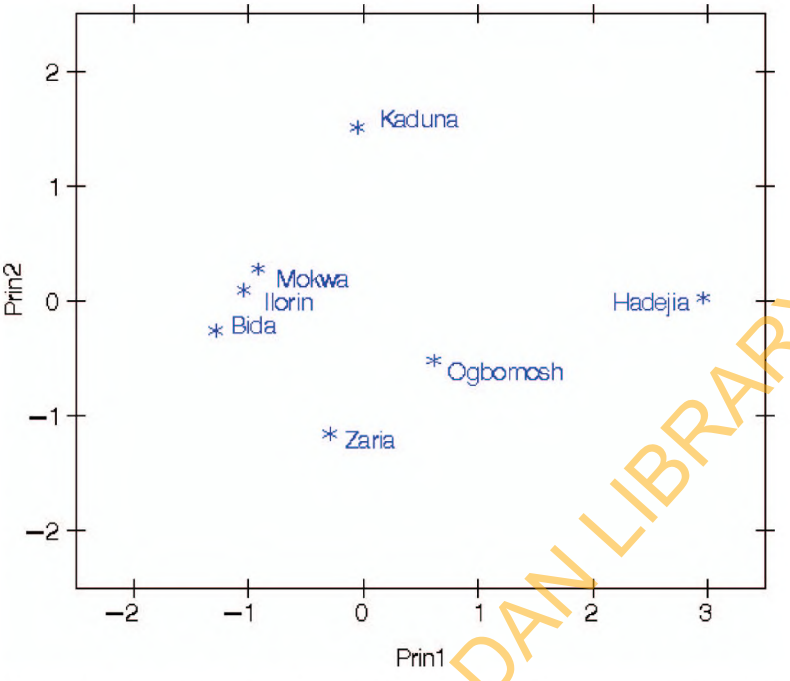

Figure 1. Biplot showing the relationship between neem seed oil extracted from different locations in the savanna AEZs of Nigeria on inhibition of radial growth of *M. phaseolina*. Where Prin = Principal component, Ogbomosh = Ogbomosho.

Discussion

Neem seed oil extracted from seeds collected from different savanna AEZs of Nigeria differed significantly in respect of their *in vitro* effects on two cowpea fungal pathogens, *Macrophomina phaseolina* (*Macrophomina* blight) and *Collectotriclium capsici* (brown blotch). Thus, their effects on mycelial growth of the two fungi on APDA as well as their minimum inhibition, concentrations and overall rating of efficacy were significantly different from one another. These differences are not attributable to variation in the latitudinal location of the seed collection sites since oil extract from seeds collected from Hadejia in Sahel savanna and Oghomosho (in derived savanna) reduced the *in vitro* radial mycelial growth of both fungi. Neem trees occur abundantly in northern Nigeria. As shade and aesthetic trees, they line many streets in towns; they are frequently used in shelter belts and as shade trees in farms. Neem trees produce large quantities of seeds that drop and decay, apparently with little or no value to farmers. However, neem seed oil is very easy to produce (The Henry Doubleday Research Association 1999) and it can be produced locally like groundnut oil and used as an effective natural pesticide against pest and pathogens as has been reported by earlier workers (e.g. Ivbijaro 1990, 1993; Obi 1993; Lale and Abdulrahman 1998). In the present study, there was no clear trend in relationship between the *in vitro* toxicity of neem seed oil to the two fungal pathogens and latitudinal location of the seed collection sites. For example, the highest toxicity to *M. phaseolina* was produced by oil extracted from seed collected from Hadejia in Sahel savanna, which was followed by oil extracted from seeds collected from Ogbomosho (derived savanna), which are both at two ends of the country from wet to arid environments. Similarly, the greatest

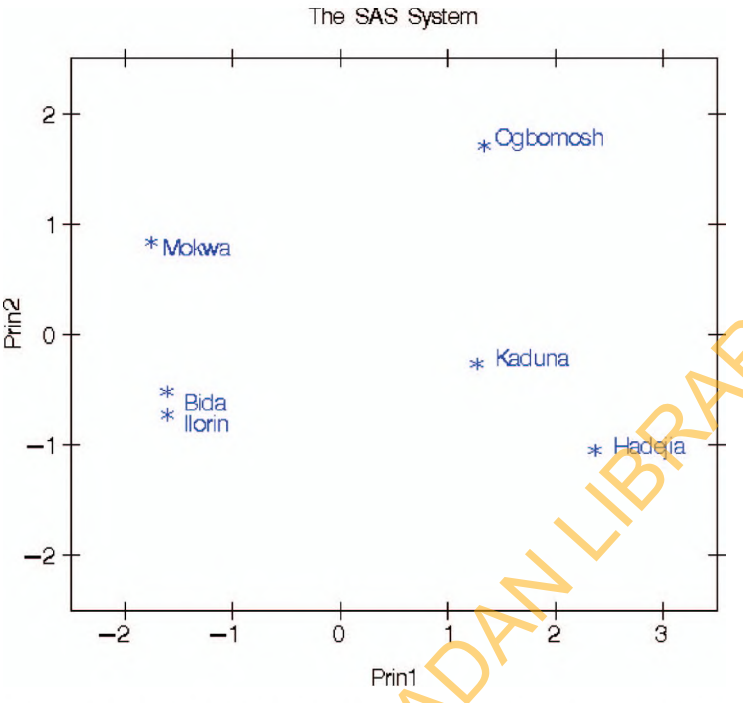

Figure 2. Biplot showing the relationship between neem seed oil extracted from different locations in the savanna AEZs of Nigeria on inhibition of radial growth of *C. capsici*. Where Prin = Principal component, Ogbomosh = Ogbomosho.

reduction in mycelial growth *in vitro* of *C. capsici* occurred in APDA containing oil from neem seeds collected at Hadejia, which was followed by oil from seeds collected at Ogbomosho. In this case, however, the next most effective oil extracts were from seeds collected from Kaduna and Zaria, in that order, both located in the northern Guinea savanna. Also, oils from seeds collected at two locations in the southern guinea savanna (Mokwa and Bida) and one location in the derived savanna (Ilorin) have no effect on *M. phaseolina*. However, they were effective or moderately effective on *C. capsici*. It appears that the fungal toxicity of oil extracted from neem seeds is location specific and is probably associated with ecotype or variety or strain of neem grown in these environments as suggested by Dales (1996) and Pamplona and Roger (1999). This is probably the first report of a study in which the fungal toxicity of neem seed oil was related to the geographical location of the trees that produced the seeds. The results suggest that it may be necessary to evaluate the relative efficacy of neem seed oil from different locations in an effort to use the oil as a pesticide against specific pathogens.

Although the trends in *in vitro* toxicity of neem seed oil to two important cowpea pathogens have generally been corroborated by the results of biplot analysis, the potential of using neem seed oil in the control of cowpea diseases requires further investigation, including fungicidal trials under field conditions, both on-station and on-farm. Recently, the United Nations Industrial Development Organisation (UNIDO) has embarked on a major initiative to promote appropriate low-cost, but sustainable technologies and techniques for large-scale production of biopesticides from neem in West Africa (UNIDO, April 2007, unpublished information).

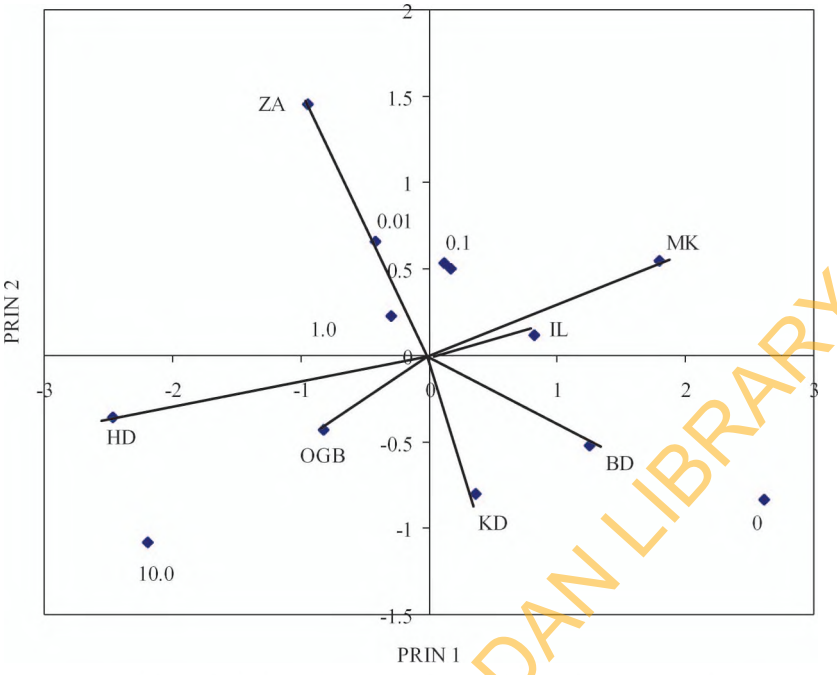

Figure 3. Biplot showing the relationship between neem seed oil extracted from different locations in the savanna AEZs of Nigeria and at different concentrations tested. Where PRIN = Principal component.

Acknowledgements

We are grateful to the International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria for financial support for this research work.

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