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In vitro and *in vivo* control of pearl millet midrib spot using plant extracts

Zarafi, A. B. and Moumoudou, U.
Dept. of Crop Protection, Faculty of Agriculture, Ahmadu Bello University, PMB 1044 Zaria

Corresponding author e-mail: afinikiz@yahoo.com
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ABSTRACT
Objective: To evaluate the efficacy of cold and hot water extracts of neem leaf and seed, mahogany seed, ginger rhizome and shea butter leaf against Curvularia eragrostidis.
Methodology and results: The efficacy of cold and hot water extracts of neem leaf and seed**,** garlic bulb, mahogany seed, ginger rhizome and shea butter leaf was tested in the control of *Curvularia eragrostidis* isolated from pearl millet in *vitro* and *in vivo*. Twenty grams each of ethanol sterilized plant material was either suspended in 100 ml of sterile water for 24 hr to get cold water extract or suspended in 100 ml of sterile water and placed in a water bath at 90 ^{o}C for 1.5hr to get hot water extracts. The filtered extracts were incorporated into PDAS used to culture Curvularia eragrostidis. Radial mycelia growth, sporulation and spore length and width were determined from these cultures at 7 and 14 days after inoculation. Cold water extracts were staggered applied on potted pathogen inoculated pearl millet plants to get application at 2 days before inoculation (DBI), 2days after inoculation (DBI) and at symptom appearance time (SAP).Disease incidence and severity were assessed on these plants. The cold water extract of each tested plant material reduced mycelial growth, sporulation and spore size of the pathogen better than hot water extracts. The efficacy of garlic was significantly (P= 0.05) reduced by heat the most while that of ginger was significantly (P= 0.05) the least. Application of the cold water plant extracts 2 days before inoculation (2DBI) and 2 days after inoculation (2DAI) resulted in a significant (P= 0.05) reduction of the disease incidence and severity compared to application of plant extracts at symptom appearance time (SAP)
Conclusion and application: The conclusion of this study is that cold water plant extracts could be used successfully as environmentally safe and economical fungicides against *Curvularia eragrostidis* causal agent of pearl millet midrib spot.
Key words: Pearl millet, *Curvularia eragrostidis*, plant extracts.

INTRODUCTION
Pollution problems in the environment and toxic effects of synthetic pesticides on non-target organisms have prompted investigations on exploiting pesticides of plant origin. Natural plants products and their analogues are an important source of agricultural pesticides (Grainge and Ahmed, 1988,Chandrasekaran and Gunasekaran, 2007;) used in the control of insect pests (Emosaire and Ukeh, 1996) , plant diseases (Al-Abed *et al*., 1993) and bird repellants (Mason and Mathew, 1996). Further more, pesticides of plant origin are cheaper, readily available and cost effective in developing countries where synthetic fungicides are scarce, often adulterated and

expensive for resource poor farmers. Through *in vitro* investigations, Bankole (1994), Adetogun and Obagwu *et al*. (1997), Atayese (2006), Eziashi *et al*.(2006), Muhamad and Mustapha (2006), Akpa and Amodu (2006). Ogbebor *et al*. (2007) and Nduagu *et al*. (2007) confirmed the fungicidal potential of extracts of *Azadirachta indica (neem insert common name), Khaya senegalensis(mahogany), Allium cepa(garlic)* and *Zinziber officinale (ginger)* on *Alternaria solani, Colletotrichum* spp., *Fusarium oxysporum, Rhizoctonia solani, Penicillium corylophilim, Ceratocystis paradoxa, Drechstera heveae, Xanthomonas oryzae,* and *Erwinia carotovora* which are all pathogenic on valuable crop plants causing important diseases. Alabi (1986) reported efficacy of both cold and hot water extracts of *Azadirachta indica* on *Dothiorella dominica*, the causal agent of mango soft rot. Amadioha (2000) obtained lower rice blast (*Pyricularia oryzae*) incidence and severity with cold water extracts of *Azadirachta indica* compared to hot water and alcohol extracts. Markson *et al.* (2004) and Madunagu *et al.* (2004) also reported the superiority of cold water extracts of *Piper guineensis, Aframonum melegueta, Jatropha curcas, Ageratum conzyoides* and *Emillia sanchifolia* over their ethanolic extracts. Much of the plant kingdom still remains unexplored for possible exploitation against major fungal pathogens such as *Curvularia eragrostidis*, the causal agent of pearl millet midrib spot (Zarafi *et al*., 2004). *Azadirachta indica* (neem), *Khaya senegalensis* (mahogany) and *Vittelloria paradoxa* (shea butter) are traditionally used as local medicines (either as water extracts or as oils) in Nigeria and other African countries. The purpose of this study was to determine the efficacy of cold and hot water extracts of neem leaf and seed (*Azadirachta indica*), garlic bulb (*Allium sativum*), mahogany seed (*Khaya senegalensis*), ginger rhizome (*Zingiber officinale*) and shea butter leaf (*Vitellaria paradora*) extracts on *Curvularia eragrostidis*.

MATERIALS AND METHODS

Pathogen isolation: *Curvularia eragrostidis* was isolated in the laboratory from an infected pearl millet midrib that had demonstrated typical disease symptoms in the field at Basawa, Zaria. The infected midrib portions were cut into 2 mm pieces, surface sterilized with 0.5% solution of sodium hypochlorite and rinsed in three changes of sterile water. Four pieces were placed per Petri dish containing 20 ml potato dextrose agar (Merck KGaA, Darmstadt, Germany) amended with streptomycin (PDAS) and then incubated at 28 $\pm$ 2^{o} C for 5 days. The pure culture of *Curvularia eragrostidis* was maintained in PDAS slants until needed.

Preparation of plant extracts and their in vitro evaluation: The various plant materials were air-dried, powdered separately using mortar and pestle and then blender (Benatone blender model BLG – 40L).Twenty grams of each powder was mixed with 5 ml 95% ethanol for 5 minutes for surface sterilization. The cold-water extracts were obtained by infusing 20 g of each powder in 100 ml of sterile water for 24 hours. The hot water extracts were prepared by mixing 20 g each of sterilized plant material in 100 ml of sterile water and each flask was placed in a water bath at 90^{o} C for 1.5 hours.

The suspensions (cold and hot water) were filtered separately through a double layer of sterile muslin cloth. Ten milliliters of each suspension was mixed in 100 ml of PDAS to obtain potato dextrose plant extract agar (PDAS–plant extracts). Petridishes were dispensed with 20 ml of PDAS – plant extract and allowed to solidify. Inoculum discs of 4 mm diameter obtained from the edge of a seven day old culture of *C. eragrostidis* on PDAS were inoculated face downwards at the centre of each of the different plant extract plates. PDAS without any plant extract served as control, while PDAS with benomyl served as standard check. Each treatment had five replicates (5 Petri dishes) which were kept in a laboratory bench at room temperature (28 $\pm$ 2^{o}C) using completely randomized design (CRD). Mycelial growth was determined by measuring culture size along two diameters at 7 and 14 days after inoculation (DAI). At 14 DAI each Petri dish was harvested in 150 ml sterile water by blending and sieving through double layer muslin cloth. Spore count and spore measurement were also done, by means of three haemocytometer readings recorded for each replicate. For each treatment, spore length and width of 50 randomly selected spores was measured. Data

collected was subjected to T-test analysis using SPSS/PC package.

Effect of extracts on disease incidence and severity: The effects of the extracts on disease development and spread were determined using potted plants in the green house. Seeds of zango millet (local variety) were surface sterilized in 0.5% solution of sodium hypochloride solution for a minute, rinsed with sterile water and 3 seeds / pot were sown in 23 cm diameter pots containing 4 kg previously heat-sterilized loam soil. Three weeks after emergence, the regularly watered (with tap water) potted plants were randomly arranged into three groups in a glasshouse. The plants in each group were spray inoculated with a spore suspension ($5x10^5$ per ml) obtained from a 7 day old culture of *C. eragrostidis*. Application of plant extracts on these three groups of potted plants was staggered to get application at 2 days before inoculation (DBI), 2 days after inoculation (DAI) and symptom appearance time (SAP). In each group, plants sprayed inoculated with the pathogen only served as control while those treated with benomyl served as standard check. The disease incidence was determined by counting diseased leaves and expressing it as a percentage of the total number of leaves in the plants / pot. Disease severity was recorded on a 1 to 5 scale, where, 1 = No mid rib infection; 2 = 1-20 % of leaf midrib infected; 3 = 21- 40 %; 4 = 41-60 % and 5 = 61-100 % leaf midrib infected. The Severity formula used was

$\Sigma \frac{nx}{N} (5)$ (Chaube and Pundhir, 2005),

where x = grade per leaf;
n = number of leaves per given grade,
N = total number of leaves examined/pot;
5 = the maximum disease grade.

Five replications were maintained for each treatment in the two separate experiments. Data collected were subjected to analyses of variance (ANOVA) and means were separated using Least Significant Difference LSD at 5% level of significance.

RESULTS AND DISCUSSION:

At 7 DAI, (Table 1) the plant extracts inhibited mycelial growth of *C. eragrostidis* compared to control although none was as inhibitory as the synthetic fungicide (benomyl).Among the plant extracts, garlic had the highest (P= 0.05) inhibitory effect while ginger had the least, both as cold and as hot water extracts. For each plant material, the inhibitory effect of cold water extract was significantly higher (P= 0.05) than hot water extract except for ginger where the difference between hot and cold water extracts was not significant (P= 0.05). At 14 DAI, a similar trend was observed, where hot water extracts still had lower inhibitory effect, suggesting that the fungistatic components of these plant extracts are denatured by heat (Figure 1 and Table 1).

Table1: Effect of cold and hot water plant extracts on mycelial growth of *Curvularia eragrostidis*

Extraction method	Mycelial growth (cm) on PDAS amended with:							
	Garlic	Shea butter leaf	Neem leaf	Neem seed	Mahogany seed	Ginger	Benomyl	Control
				7DAI				
Cold water	2.73 b	5.56 b	4.44 b	4.01 b	4.85 b	6.10 a	3.66 a	9.00 a
Hot water	6.65 a	8.00 a	6.42a	5.71 a	6.53 a	6.81 a	3.66 a	9.00 a
Table t- value					2.98			
				14DAI				
Cold water	4.02 b	9.00a	5.71 b	6.01 b	7.08 b	9.00 a	6.61 a	9.00 a
Hot water	8.88 a	9.00 a	8.72 a	8.10 a	8.74 a	9.00a	6.61 a	9.00 a
Table t-value					2.98			

Values in the same column carrying the same letter(s) are not significantly different (P=0.05) using t –Test

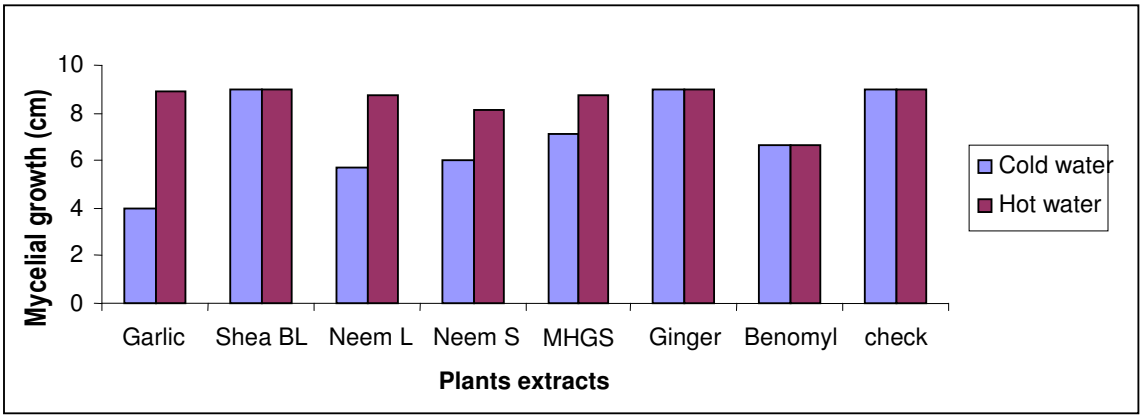

Figure1: Effect of cold and hot water extracts on mycelial growth at 14DAI.Key: Shea BL=she butter leaf; MHGS= mahogany seed; S=seed; L=leaf

However, the cold water ginger extract (9.00 cm) and hot water ginger (9.00 cm) did not differ, also shea butter cold (9.00 cm) and hot water (9.00 cm) extracts did not significantly (P= 0.05) differ from each other. Amadioha (2000) similarly reported high efficacy of cold water neem leaf extracts compared to hot water extracts. Lubna and Husham (2007), also reported that exposure of neem extracts to high temperatures (55^{o}C and above) caused most of the components to disappear or their concentration significantly decreased. They also reported that the components are most effective and stable at 25 to 35^{o}C and pH of 6.5 to 8.6. Exposure to sunlight and UV light was also found to be detrimental to the neem extracts. Among the plant material tested, garlic was heat-denatured more compared to other plant materials as evidenced by wide difference between the mycelial growth of hot and cold water garlic extracts. Markson et al. (2004) reported the superiority of cold water extracts over the ethanolic extracts. However, the fungitoxicity of the plant extracts did not persist over 10 days, contrary to the finding of this study which the efficacy of plant extracts were visible even at 14 DAI. Alabi (1986) recorded higher inhibitory effect of bark neem extracts on mycelial growth and sporulation of *Dothioriella dominica* than the leaf extracts. In this study, neem leaf extract was not as inhibitory as the neem seed extract. Obagwu et al. 1987, in an independent study, obtained significantly high inhibitory effect on mycelial growth and sporulation of *Coletotrichum capsici* from garlic water extract. Water extracts of *Allium* sp. gave high inhibition of mycelial growth followed by *A. indica*. Ogbebor et al (2007). Similarly, in this study, *Allium cepa* performed better than neem and other plant extracts. The cold water plant extracts also inhibited sporulation higher than hot water extracts (Figure .2 and Table 2).

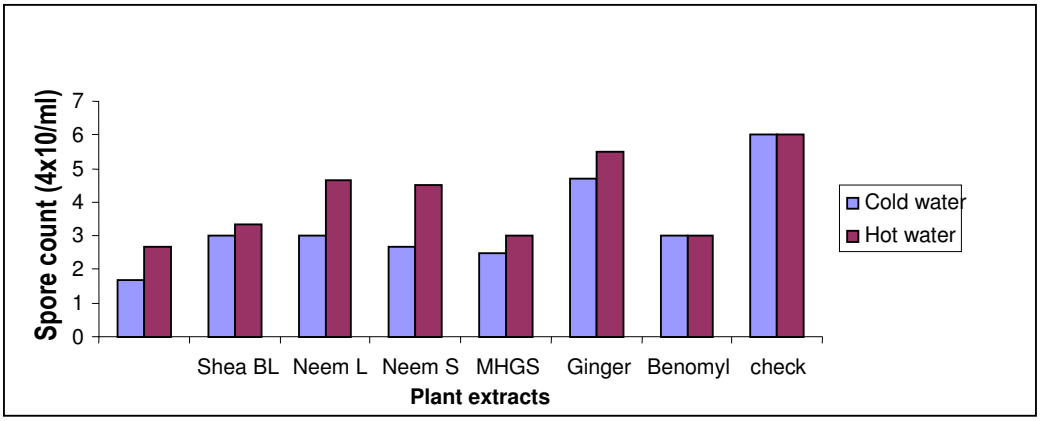

Figure 2: Effect of cold and hot water extracts on sporulation at 14 DAI
Key: Shea BL=she butter leaf; MHGS= mahogany seed; S=seed; L=leaf

Table 2: Effect of cold and hot water plant extracts on sporulation and spore size of *Curvularia eragrostidis*

Extraction method	Sporulation and spore size (um) grown on PDAS amended with:							
	Garlic	Shea butter leaf	Neem leaf	Neem seed	Mahogany seed	ginger	benomyl	control
	Spore count (x 10^4 / ml)							
Cold water	1.67[b]	3.00[b]	3.00[b]	2.67[b]	2.50[b]	4.70[b]	3.00[a]	6.00[a]
Hot water	2.67[a]	3.33[a]	4.67[a]	4.50[a]	3.00[a]	5.50[a]	3.00[a]	6.00[a]
Table t-value	2.62							
	Spore length (um)							
Cold water	0.65[a]	0.66[a]	0.71[a]	0.67[a]	0.65[a]	0.73[a]	0.71[a]	0.80[a]
Hot water	0.67[a]	0.73[a]	0.79[a]	0.74[a]	0.73[a]	0.74[a]	0.71[a]	0.80[a]
Table t-value	0.85							
	Spore with (um)							
Cold water	0.30[a]	0.31[a]	0.32[a]	0.29[a]	0.29[a]	0.32[a]	0.33[a]	0.35[a]
Hot water	0.33[a]	0.34[a]	0.34[a]	0.35[a]	0.31[a]	0.32[a]	0.33[a]	0.35[a]
Table t-value	0.85							

Values in the same column carrying the same letter(s) are not significantly different (P=0.05) using t –Test

Spore size (spore length and width) however, did not differ significantly (P= 0.05) for the hot and cold water extracts of all the plant materials used in this experiment. Among the plant extracts tested, garlic (*Allium cepa*) induced the lowest number of spore production which also had the smallest sizes, while ginger also had the lowest effect on both sporulation and spore size. In the *in vivo* testing of cold water extracts, garlic controlled/ reduced the disease more than the other plant materials, although the difference was not statically significant (P=0.05) in all the plant material application time (Tables 3 and 4).

Table3: The effect of cold water plant extracts on midrib spot disease incidence

Plant extract	Disease incidence (%) when plant extracts were applied at:		
	2DBI	2DAI	SAP
Garlic	0.82[abc]	0.62[a]	0.93[ab]
Neem leaf	2.00[bc]	2.01[ab]	2.51[ab]
Neem seed	1.60[abc]	1.40[ab]	2.11[ab]
Mahogany seed	0.61[ab]	0.99[ab]	1.49[ab]
Shea butter leaf	1.77[abc]	2.04[ab]	2.32[ab]
Ginger	2.26[c]	2.53[ab]	3.04[b]
Benomyl	0.35[a]	0.51[a]	0.71[a]
Control	5.97[c]	5.97[c]	5.97[c]

DBI = days before inoculation; DAI = days after inoculation; SAP = at symptom appearance
Values in the same column carrying the same letter(s) are not significantly different (P=0.05) using LSD.

Table 4: The effect of cold water plant extracts on midrib spot disease severity

Plant extract	Disease severity index when plant extracts were applied at:		
	2DBI	2DAI	SAP
Garlic	0.03a	0.02a	0.05ab
Neem leaf	0.22cd	0.22cd	0.21cd
Neem seed	0.17c	0.17c	0.16c
Mahogany seed	0.11b	0.10b	0.10b
Shea butter leaf	0.21cd	0.21cd	0.20cd
Ginger	0.28e	0.48e	0.46e
Benomyl	0.02a	0.00a	0.01a
Control	0.93f	0.68f	0.78f

DBI = days before inoculation; DAI = days after inoculation; SAP = at symptom appearance
Values in the same column carrying the same letter(s) are not significantly different (P=0.05) using LSD

Except for ginger, the plant extracts were as effective as benomyl. Ginger, although not as effective as benomyl and other plant materials, was also able to significantly (P=0.05) reduce the disease incidence and severity at all the tree application times compared to control/ untreated check. For each plant material, disease incidence recorded on plants sprayed at SAP was higher than on those plants sprayed at 2DBI and 2DAI. Similarly to this finding, disease incidence and severity of rice blast was higher in plants sprayed with neem extracts at SAP compared with those sprayed at 2DBI and 2DAI (Amadioha,2000).

CONCLUSION

These plant materials could be used as protectants applied as seed dressing fungicides or foliar spray of young plants since millet is a tall crop. However, further evaluation of the materials is needed. Also needed is the determination of the most economical rate, formulation / packaging and method of application.

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