ISSN 1996-3351

Asian Journal of Biological Sciences

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Asian Journal of Biological Sciences 4 (3): 306-311, 2011
ISSN 1996-3351 / DOI: 10.3923/ajbs.2011.306.311

Evaluation of Antifungal Potential of *Ocimum gratissimum* Extracts on Two Seedborne Fungi of Rice (*Oryza sativa* L.) in Cameroon

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ABSTRACT

A study was undertaken to investigate the antifungal effect of essential oil and solvent extracts of dried leaves of *Ocimum gratissimum* L. in the quest for a possible bio-fungicide. Previous studies unveiled, rice farming regions of Cameroon were endemic to *Bipolaris oryzae* and *Alternaria padwickii* causal of brown-spot and stack-burn diseases respectively. A comparative *in vitro* study exploring the antifungal effect of *Ocimum gratissimum* L. dried leaves extracts and a local synthetic fungicide (Chlorothalonil 550 and 100 g L^{-1} Carbendazim referred to as Banko-plus) against these two phytopathogens using the supplemented medium technique was setup. Essential oil obtained through hydrodistillation stood at pars from ethanol extract, cold water extract, hot water extract and exhibited the best antifungal activity on *Bipolaris oryzae* and *Alternaria padwickii* at (150 ppm), marked by 86.17 and 100% growth inhibition, respectively. The synthetic fungicide strongly inhibited *B. oryzae* and *A. padwickii* at 10,000 ppm with 69.11 and 100% growth inhibition, respectively. Strong resistance was exhibited by *B. oryzae* against Banko-plus at lower concentrations, shedding light on the inefficiency of farmers to control brown-spot disease with this systemic fungicide in Cameroon. Ethanol and cold water exhibited some exploitable antifungal activity. Hot water extract showed no inhibitory activity at all tested concentrations, but paradoxically activated growth at all tested concentrations. This study paved the path for further field studies towards the exploitation of the antifungal potentials of *Ocimum gratissimum* L. essential oil, ethanol and cold water extracts for seed treatment in Cameroon rice fields.

Key words: *Ocimum gratissimum, A. padwickii, B. oryzae,* bio-fungicide

INTRODUCTION

FAO (2009) forecast of global paddy production has been lifted by nearly 10 million tonnes to 678 million tonnes, 2% below 2008's crop, but still the second highest production on record. By contrast shortfalls in Cameroon domestic rice supply had led to an annual 44% rice importation and a foster price increase (FAO, 2009). One of the main reasons for the low productivity is the endemic presence of seed borne brown-spot disease (caused by *B. oryzae)* and stack-burn disease (caused by *A. padwickii*) in the main rice fields in Cameroon (Nguefack, 2005). Like elsewhere, losses had been decreased by the use of resistant varieties, crop rotation, sanitation practices, chemical fungicides (Knight *et al.*, 1997). The disturbing cases of stack-burn disease and brown-spot disease resistance to chemical fungicide let to the formulation of a double impact systemic Banko-plus

(Chlorothalonil 550 and 100 g L^{-1} Carbendazim) widely used in Cameroon and in the world at large. Despite the used of Banko-plus, persistent resistance and low yield still prevails. Public opinion on deterioration of the environmental quality and human health (Cutler and Cutler, 1999; Dmello *et al.,* 1998) and damaged to soil beneficial organisms (Hall, 1984; Hayes and Laws, 1991) associated with chemical fungicide; the need for a cheap, durable and sustainable bio-fungicide is growing within the local farmers.

Much curiosity has been devoted to the Lamiaceae family, especially *Ocimum gratissimum* L. considered as one of the main sources of potential active metabolites. It was previously demonstrated through *in vitro* test that essential oil of *Ocimum gratissimum* had antifungal activities (Amvam-Zollo *et al.*, 1998; Lemos *et al.*, 2005) and Nguikwie (2004) showed *O. gratissimum* L. essential oil fractions had antifungal activities on *Bipolaris oryzae* and *Alternaria padwickii*. In our search for natural agrochemicals of plant origin, *O. gratissimum* L. was chosen for its availability, low cost and its high versatile antimicrobial spectrum. Moreover, despite the exploitability of *O. gratissimum* L. essential oil, little or no veritable exploitation of the solvent extract had been done. In this work, we carried out an *in vitro* comparative evaluation of the antifungal potentials of *O. gratissimum* L. dried leaves extracts with respect to Banko-plus (carbendazim 100 g L^{-1} and chlorothalonil 550 g L^{-1}) on *B. oryzae* and *A. padwickii*, in order to have precluding profiling views on the possible recommended *O. gratissimum L.* extracts to be use for field trials and qualify the sensitivity of the two pathogens to Banko-plus.

MATERIALS AND METHODS

Fresh leaves of *O. gratissimum* L. were harvested at Messassi, Yaoundé-Cameroon during the monsoon period of June 2007 and air-dried at room temperature. The latter were crushed into fine powder and 100 g each were soaked and stirred in 600 mL of cold distilled water, 600 mL of hot water (100°C) and 600 mL of 75% ethanol for 1 h, respectively. The extracts were subsequently filtered through a double fold cheese cloth. The filtrates were subjected to centrifugation at 6000 rpm for 15 min and the supernatant were lyophilized to give dry mass of Cold Water Extracts (CWE), Ethanol Extract (EE) and Hot Water Extracts (HWE), respectively. Essential oil (EO) was obtained through hydrodistillation using the Clevenger apparatus as described by Lamaty *et al.* (1987). Each plant extract was fractionated using one dimensional thin layer chromatography (TLC); with a silica gel G_{60}-alumina backed plate (10×10 cm). Cold water, hot water and ethanol extracts solutions prepared at 500 μg mL^{-1} and essential oil were applied and the layer developed with n-hexane/acetylacetate (HA) 4:1. TLC was triplicated and spots were detected in an iodine vapor chamber. The *B. oryzae* and *A. padwickii* were isolated in the laboratory from infected rice seeds as describe by Hang *et al.* (1999) and the organisms were subcultured to obtain a pure culture on a PDA medium.

Inhibitory effect of *O. gratissimum* L. extracts and Banko-plus on radial growth were determined by growing the fungi on the yeast extract agar peptone medium using the supplemented medium technique described by Grover and Moore (1962). Dishes were sealed with a parafilm paper and incubated at an inverted position (with their bottom facing light) at 24±2°C, under 12/12 h alternate cycles of near ultra-violet light and darkness for one and two weeks for *B. oryzae* and *A. padwickii*, respectively. Fungitoxicity was recorded in terms of percentage colony inhibition and qualified with Pandey *et al.* (1982) formulae as thus:

$$I\% = Ds - Dn \times \frac{100}{Dn}$$

where, I% is Percentage of radial growth inhibition, D_n is average radial growth diameter of fungus colony in non supplemented medium. D_s is average radial growth diameter of fungus colony in supplemented medium.

A pathogen was assigned as sensitive when the radial growth in the presence of Banko-plus is less than 10% of radial growth in the absence of Banko-plus; moderately resistant with 10 to 60% radial growth and resistant when greater than 60% radial growth at 10,000 ppm as defined by Koh *et al.* (1994). Fungicidal and fungistatic activity of extracts and Banko-plus fungicide against *B. oryzae* and *A. padwickii* were evaluated as described by Bindu *et al.* (1998) and Mishra and Kishore (1989), respectively. Data were analyzed using Statistical Package for Social Science (SPSS) 10.1 for the determination of mean and significant differences. The following statistical tests were applied: (1) One way ANOVA and (2) t-test student-newman-keuls (parametric) for the evaluation of the smallest significant difference with $p<0.05$.

RESULTS AND DISCUSSION

Percentage radial growth inhibition of *B. oryzae* and *A. padwickii* by *Essential oil* (EO) after 14 days of incubation on yeast extract agar peptone medium is shown in Table 1 marked with significant inhibition observed at 200 ppm, but failed to exhibit fungicidal activities. Banko-plus (composed of Chlorothalonil 550 and 100 g L^{-1} Carbendazim) exhibited a strong inhibition against *A. padwickii* than *B. oryzae* at higher concentrations. Radial growth inhibition of *A. padwickii* and *B. oryzae* on supplemented yeast extract agar peptone medium by Cold Water Extract (CWE), Hot Water Extract (HWE) and Ethanol Extract (EE) is shown in Table 2. A significant inhibition at 10,000 ppm was exhibited by EE and no fungicidal activities were observed. Characteristic physical properties of *O. gratissimum* L. extracts following extraction and one dimensional TLC are displayed in Table 2. The density of EO stood at 0.884 g mL^{-1}.

The extraction yield of EO of *O. gratissimum* L. stood at 1.46%. This result is inferior to the 1.71%, obtained by Nguikwie (2004) from plants harvested in the month of July at Obala-Yaoundé, Cameroon. This yield disparity can be related to the month and place of harvest. Yields obtained with solvents were 15.40, 9.39 and 4.49% for HWE, CWE and EE, respectively. Yield and aspect differences between HWE and CWE rely on the thermal agitation effects which increase the hydrosolubility of constituents such as starches, polypeptides and lectins in water (Cowan, 1999).

Table 1: Percentage radial growth inhibition of *B. oryzae* and *A. padwickii* by essential oil of *O. gratissimum* L. and Banko-plus (Chlorothalonil 550 and 100 g L^{-1} Carbendazim) after 14 days of incubation

Concentration (ppm)	Percentage inhibition by essential oil		Concentration (ppm)	Percentage inhibition by Banko plus (Chlorothalonil 550 and 100 g L^{-1} Carbendazim)	
	B. oryzae	*A. padwickii*		*B. oryzae*	*A. padwickii*
50	36.02±2.35^a	37.79±5.70^a	5	0.00±0.00^c	18.52±1.70^a
100	54.58±5.38^b	79.14±4.47^b	25	0.00±0.00^c	100±0.00^b
150	86.17±1.02^c	100±0.00^c	1000	69.11±2.85^a	100±0.00^c
200	100±0.00^c	100±0.00^c	2000	86.33±3.15^b	100±0.00^c
300	100±0.00^c	100±0.00^c	3000	100±0.00^c	100±0.00^c

a-c: Values in rows followed by different letters are significantly different ($p<0.05$). Mean±SD for 3 experiments

Table 2: Percentage radial growth inhibition of *B. oryzae* and *A. padwickii* in the presence of CWE, HWE and EE after 14 days of incubation and physical characteristics of *O. gratissimum* extracts triplicates Rf values

	Physical properties			Percentage inhibition (testing concentrations)		
Extracts	Yield (%)	Number of spots	R_f values	5,000 ppm	10,000 ppm	Pathogenic agent
EE	4.47	7	0.94	79.26±0.84[a]	80.92±2.84[a]	*B. Oryzae*
CWE	9.34	0	0	23.63±1.93[b]	42.95±5.72[b]	
HWE	15.4	0	0	0.00±0.00[c]	0.00±0.00[c]	
EO	1.46	6	0.97	NT	NT	
EE				59.66±0.03[a]	61.54±4.53[a]	*A. padwickii*
CWE				0.00±0.00[b]	0.00±0.00[b]	
HWE				0.00±0.00[c]	0.00±0.00[c]	

Values in rows followed by different letters are significantly different ($p<0.05$). Mean of values±standard deviation for 3 experiments. NT: not tested. R_f is the average of three repetitive TLC

The Minimum Inhibitory Concentration (MIC) of EO was obtained at 200 and 150 ppm for *B. oryzae* and *A. padwickii*, respectively on yeast extract agar peptone medium, not corresponding to the 800 and 600 ppm against *B. oryzae* and *A. padwickii*, respectively from Nguikwie (2004) working on PDA medium. The variability in MIC and efficiency of EO could be attributed to the differences in their chemical composition, which varies with the geographical location, time of harvest and plant part collected (Ntezurubanza *et al*., 1987; Menut and Valet, 1985) and medium of culture.

At par from essential oil, significant fungistatic inhibition of radial growth of 80.92 and 61.54% was observed with EE at 10,000 ppm on *B. oryzae* and *A. padwickii*, respectively. This high activity is associated to the rich phenolic groups generally found in the ethanol extract according to Bever (1986), reflecting the high number of spots and Rf values as reveal by TLC. In this regard, CWE exhibited 42.38% growth suppression uniquely on *B. oryzae*, having no antifungal activity on *A. padwickii*. Curiously, HWE unlike CWE had no antifungal effect vis-à-vis *B. oryzae* and *A. padwickii* neither at 10,000 ppm nor at 5,000 ppm, but promoted a rapid growth of both fungi. In this light, it acted as manure or better still, a growth activator. This can be explain by the fact that, constituents of aqueous extracts responsible for inhibitory activities such as polypeptides and lectins are denatured by the heating process at the course of extraction according to Cowan (1999).

These results obtained on fungitoxicity potential of the solvent extracts is in accordance with those of Amadioha (2000) from his studies on controlling rice blast *in vitro* and *in vivo* with extracts (such as CWE, HWE, EE and EO) of *Azadirachta indica*. This author outlined in decreasing order of antifungal activity EO, EE, CWE and HWE of *Azadirachta indica* on *Pyricularia oryzae* which tie-up with our results. Edeoga *et al*. (2006) after phytochemical analysis pointed out that *O. gratissimum* L. leaves are rich in chemical bases such as alkaloids, flavonoids, saponins and tanins. Nontheless, disparity in extract activity of same plant is influenced by several factors such as method of extraction, age of plant, time of harvest and different extracting solvents (Nicolls, 1969; Menut and Valet, 1985). Characterization of *O. gratissimum* L. EE revealed the presence of non-cyclic sesquiterpenes, phenols (Esvanszhhuga, 1986), eugenol, alpha-pinen, camphor and terpinene (Bever, 1986). Moreover, most inhibitors extracted are not water soluble as in the case of water-extraction, where isolation of active components depends on hydrosolubility (Cowan, 1999). This could explain the superiority of EE over aqueous extracts.

TLC revealed that CWE and HWE spots did not migrate in the n-hexane/acetylacetate 4:1 non-polar mobile phase. This, further confirmed constituents of water extracts are mostly polar. TLC results also unveiled that active components found in EO and EE are non polar in nature with respect to their high R_f values. Furthermore, Menut and Valet (1985) and Amvam-Zollo *et al.* (1998) reported a higher content of thymol in the EO of *O. gratissimum* L. (46.5%) highly responsible for antimicrobial activities. Ultee *et al.* (2002) suggested that, the activity of thymol (46.5% present in EO of *O. gratissimum* L.) is attributed to the characteristic feature of the phenolic hydroxyl group and the presence of a system of delocalized electrons. This explains the overall superiority of EO antifungal potential over the solvent extracts. Moreover, the high antifungal potency of EO can be attributed to the membrane destabilization, decrease in membrane potential and decrease in delta pH as suggested by Ultee *et al.* (2002). Phytochemical views were further strengthened by analytic TLC results, which revealed the highest R_f value (of 0.96) associated with EO, implying that its antifungal constituent are more apolar than those of EE. Hence, the number of spots and high R_f values are good reflections of activity and number of molecules present in each *O. gratissimum* L. extract.

Bioassay with Banko-plus appeared to be very efficient on *A. padwickii* (with MIC of 25 ppm) compare to *B. oryzae* (with MIC of 3,000 ppm), implying that *B. oryzae is* resistant to Banko-plus to a certain degree. This result correlates with findings of Ahmed *et al.* (2002), that non specific fungicides to *B. oryzae* are effective only at higher concentrations. Hence, *B. oryzae* is resistance to Banko-plus following Koh *et al.* (1994) grading scale. The efficacy of EO on *B. oryzae* was very high compared to the fungitoxicity of Banko-plus, which in return had a high inhibitory activity on *A. padwickii* compared to EO. Also, EO completely inhibited both fungi at 200 ppm, whereas complete inhibition with Banko-plus were observed at 3,000 ppm and 25 ppm for *B. oryzae* and *A. padwickii*, respectively. This clearly demonstrates pathogenic resistance of *B. oryzae* to the systemic fungicide. Amazingly, EE at 10,000 ppm exhibited an 80.92% inhibition on *B. oryzae*, whereas only 69.11% inhibition of the latter was observed with Banko-plus. This glaring evidence gives green light on the possible usage of ethanol extract or essential oil of *O. gratissimum* L. as biofungicide for the treatment of rice seeds by the local farmers for controlling brown-spot diseases. However, for this to be possible, *in vivo* field stability test, fungitoxicity and phytotoxicity tests must be carried out.

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