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Use of Three Essential Oils as Seed Treatments Against Seed-borne Fungi of Rice (*Oryza sativa* L.)

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Abstract: Three essential oils (EOS) extracted from *Cymbopogon citratus*, *Ocimum gratissimum* and *Thymus vulgaris*, were tested for their ability to control seed-borne infection and seed to seedling transmission of *Alternaria padwickii*, *Bipolaris oryzae* and *Fusarium moniliforme* in naturally infected seeds. Seven rice cultivars were tested and the EOs applied as slurry controlled the seed infection at a range of 48% to 100%. The treatment also reduced the seed to seedling transmission of the referred fungi at a range of 76% to 95%. The vigour of rice seedlings raised from the treated seeds was better compared to that of seedlings from the untreated seeds. The three Eos also increased the germination capacity of the treated seeds with 5 to 13%. On-farm evaluation is presently in progress in Cameroon.

Key words: Essential oils · Rice · Seed-borne fungi · Seed treatments

INTRODUCTION

Rice (*Oryza sativa* L.) is the third important cereal crop in the world after wheat and maize, with an annual production of 576 million tonnes (with 90% in Asia, 5% in America and 3% in Africa) that should be increased by 75% by 2025 to satisfy the growing population requirement estimated at 850 million tonnes of paddy [1-3]. As other African countries, rice has become a staple food in Cameroon and each citizen consumes a mean of 12.7-20.0 Kg of rice per year. However the national rice production is declining continuously and passes from 107 000 tons in 1985 to 55 000 tons in 2003. In contrary rice importations are increasing and rose from 152 030 tons in 1999 to 250 498 tons in 2001 [1]. Consequently, Cameroon is relying on foreign rice to satisfy the growing population requirement. The weakness of this sector and its abandon by farmers are due many constraints among which, infertility of soils, prevalence of pests and disease infestations, poor cultural practices [2, 24, 37]. According to Agarwal *et al*. [4], Dharam Vir *et al*. [6], Nsemwa and Wolffhechel [7] the most important and widely distributed seed-borne fungal diseases of rice are blast (*Pyricularia oryzae)*, brown spot (*Bipolaris oryzae*), bakanae (*Fusarium moniliforme*) and stackburn disease (*Alternaria padwickii*). Such seed-borne infections are known to decrease the germination of rice seeds [8, 9]. To this effect, Agarwal *et al*. [4] and De Waard *et al*. [2], suggested disease management through seed treatments as an integral component of rice crop production to avoid unacceptable crop losses and also to meet the challenge posed by feeding the growing world population. Dharam Vir *et al*. [6], Bateman *et al*. [10] and De Waard *et al*. [2] have reported the use of fungicides in controlling diseases of rice. Nevertheless, agrochemicals are not widely used by small-scale rice producers due to the non availability and high cost of suitable products [11, 5]. Furthermore, obvious pollution of the environment and the toxic effects of synthetic chemicals on non-target organisms including humans have prompted investigations on pesticides of plant origin [12]. The practical use of natural compounds as control agents is receiving increased attention and this is partly due to their non-toxicity to humans, their systemicity and biodegradability [13, 14]. Volatile compounds from plants, especially essential oils have been demonstrated to possess potent antifungal, antibacterial, insecticidal and nematocidal activity [15-21]. However, the use of essential

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oils as seed treatments for controlling seed-borne infection has been investigated on a limited number of crops [22, 23, 20, 24].

Therefore, this study on rice seed treatment with essential oils, extracted from three aromatic plants was initiated. Attention was paid to the impact of the treatment on the germination, the seed borne fungi and their seed to seedling transmission with reference to *A. padwickii*, *B. oryzae* and *F. moniliforme*.

MATERIALS AND METHODS

Rice Seed Samples: Seed samples of seven different rice cultivars were used. Five were from Vietnam (IR 50404, IR 504040, Khangdan, OM 1490 and TDNB 100) one from Bangladesh (BR-11) and one from India (local variety). They were identified during seed health testing with low (<20%), medium (20-40%) and high (>40%) infection of, *Alternaria padwickii* (Ganguly) M.B. Ellis, *Bipolaris oryzae* (Breda de Haan) Shoem and *Fusarium moniliforme* Sheldon, respectively, at the Danish Government Institute of Seed Pathology for Developing Countries (DGISP).

Essential Oils: The EOs from fresh leaves of *Cymbopogon citratus* (D.C.) Stapf*, Ocimum gratissimum* L. and freshly harvested whole plants of *Thymus vulgaris* L, were used. They were extracted by hydrodistillation using Clevenger's apparatus as recommended by Amvam Zollo *et al*. [15]. The EO recovered from each extraction was dried over anhydrous sodium sulphate and stored in darkness at 4ºC. The yields of the essential oils were as follows: 0.42%, 0.57% and 0.36%, weight of the fresh plant material, respectively for *C. citratus*, *O. gratissimum* and *T. vulgaris*. These plant species were selected on the basis of the previous knowledge on their antifungal activities [18]. The plants are grown for commercial use in the humid forest and highland agroecological zones of Cameroon and are used as food flavours and medicinal purposes.

Seed Treatments: The seeds were treated with an emulsion of EO and 0.1% water-agar solution following Remmal *et al*. [25]. The EOs were mixed at the ratio of 4:100 (v/v) for EOs from *O. gratissimum* and *T. vulgaris* and 1.5:100 (v/v) for the EO from *C. citratus*. One hundred (100) µl of the mixture was applied per gram of rice seeds according to Adegoke and Odesola [22]. These concentrations were selected based on our preliminary studies, which showed that rice seed treated with the EO emulsions applied at the above ratio had no adverse effect on their germination capacity. Two controls were used: one treated with a commercial fungicide Dithane M-45 (Zinc-manganese ethylene bisdithiocarbamate) (KVK AGRO) at the rate of 0.3% of seed weight according to Dharam Vir *et al*. [26] and another with water-agar solution without EO. The treated seeds were maintained at room temperature for 24 h in covered Erlenmeyer flasks (100 ml), before any subsequent testing.

Seed Health Testing: Seed health testing was performed using the standard blotter method described by the International Rules for Seed Testing [27]. Seed-borne fungi were identified on incubated seeds using description book of Agarwal *et al*. [4]. Two hundred seeds were tested from each cultivar and for each treatment, 4 replicates of 50 seeds. The incidence of *A. padwickii*, *B. oryzae* and *F. moniliforme* was recorded and the experiment was repeated twice.

Seed to Seedling Transmission: Another fraction of treated and non-treated seeds from each of the seven cultivars was sown in standard peat soil (Weibull K-Soil) in plastic pots in 2 replicates of 50 seeds. The pots were placed in a growth chamber at 25-30°C under cycles of 12 h day light/12 h darkness. After 14 days the seedlings were assayed for recovery of *A. padwickii*, *B. oryzae* and *F. moniliforme*. Two replicates of 30 randomly picked seedlings were evaluated for each treatment. The seedlings were taken gently from the pots and washed under tap water. They were surface sterilized in 1% sodium hypochloride for 1 minute, cut under aseptic conditions into sections of about 5-10 mm. Three sections (S1, S2, S3) from each seedling were plated for recovery of the fungi in question. The sections were described as follows: S1 (portion of the seedling from the mesocotyl), S2 (portion of the seedling on either side of the coleoptile tip), S3 (portion of the seedling on either side of the point of separation of the lamina of the first leaf). The experiment was repeated twice and the effect on the seed to seedling transmission was calculated for each fungus as the percent difference of recovery between the non-treated and treated.

Germination Test: The germination of rice seeds was performed following the conditions described by the International Rules for Seed Testing [28]. Two hundred seeds were tested in 4 replicates of 50 seeds each, using the between paper (BP) method [28]. After rolling, the blotter papers were placed in polyethylene bags and incubated at an upright position in a growth room at

28-30°C under a cycle of 12 hr light/12 hr darkness. The experiment was repeated twice.

Data Analysis: The data were subjected to analysis of variance (ANOVA) and separation of means using the least significance difference (LSD) with $\alpha < 0.05$. This was based on Sigma Stat program [5].

RESULTS

Control of Seed-borne Infection and Seed to Seedling Transmission of *A. Padwickii*: Seed treatments with EOs from *C. citratus*, *O. gratissimum* and *T. vulgaris* controlled the level of infections of *A. padwickii* in rice seed by 48%, 95% and 99% respectively (Table 1). This effect was statistically significant (P<0.05) compared to non-treated controls and EO from *T. vulgaris* and *O.gratissimum* was just as effective as Dithane M-45 (P>0.05). The effect of EO from *C. citratus* was considerable lower than the Dithane M-45 effect (Table 1).

The transmission of *A. padwickii* from seeds to seedlings was reduced significantly (P<0.05) by 76%, 79% and 85%, with the EOs from *C. citratus*, *T. vulgaris* and *O. gratissimum*, respectively and 77% with Dithane M-45 (Table 2). The fungus was recovered from seedlings of six out the seven seed samples infected with the fungus. The level of seedling infections was lower than that of seed infection except for the local cultivar from India. Despite the fungicidal effect of Eos from *T. vulgaris* and *O. gratissimum* and Dithane M-45 (0.2, 1.7 and 0.1%, respectively), seedlings from seeds of this sample treated with the same preparations disclosed higher infection level of *A. padwickii* (3.7, 2.6 and 2.6%, respectively) (Table 1, 2).

Control of Seed-borne Infections and Seed to Seedling Transmission of *B. oryzae*: Seed treatments with EOs from *O. gratissimum* and *T. vulgaris* controlled 100% seed infection by *B. oryzae* and that from *C. citratus* controlled 93% of the same infection. The effect was statistically significant (P<0.05) compared to the untreated controls (Table 1 and Fig. 1) and only EO from *C. citratus* was significantly weaker (P>0.05) than Dithane M-45.

Seed treatments with Eos from *C. citratus*, *O. gratissimum* and *T. vulgaris* reduced the seed to seedling transmission of *B. oryzae* by 91%, 86% and 83%,

Table 1: Effect of treatments with EOs and Dithane M-45 on seed infection of *A. padwickii*, *B. oryzae* and *F. moniliforme*

	Treatments				
	Non-treated	Essential oils			Fungicide
Fungi	Control	*C. citratus*	*T. vulgaris*	*O. gratissimum*	Dithane M-45
A. padwickii	33.6±17.7	17.4±13.7	0.2±0.3	1.7±1.8	0.1±0.3
	(0[e])	(48[c])	(99[a])	(95[a])	(100[a])
B. oryzae	28.0±25.6	2.1±2.8	0.0±0.0	0.0±0.0	0.7±1.0
	(0[e])	(93[b])	(100[a])	(100[a])	(98[a])
F. moniliforme	6.0±5.6	0.3±0.5	0.0±0.0	0.0±0.0	0.0±0.0
	(0[e])	(95[a])	(100[a])	(100[a])	(100[a])
Mean reduction	0	78.6	99.6	98.3	99.3

( ) Reduction percentage in seed infection recorded in Blotter Test

[a]: Values in the same row followed by different letter are significantly different (P<0.05). Data are Means±SD of seven, six and five replicates for *A. padwickii*, *B. oryzae* and *F. moniliforme*, respectively. Each replicate is average of four recordings from two separate experiments

Table 2: Effect of treatments with EOs and Dithane M-45 on seed to seedling transmission of *A. padwickii*, *B. oryzae* and *F. moniliforme*

	Treatments				
	Non-treated	Essential oils			Fungicide
Fungi	Control	*C. citratus*	*T. vulgaris*	*O. gratissimum*	Dithane M-45
A. padwickii	17.5±17.7	4.2±3.3	3.7±3.2	2.6±3.9	2.6±3.9
	(0[e])	(76[b])	(79[b])	(85[b])	(85[b])
B. oryzae	11.1±11.8	4.0±5.5	1.0±1.6	1.9±2.2	1.6±1.7
	(0[e])	(91[a])	(83[b])	(86[b])	(64[c])
F. moniliforme	15.2±9.7	1.6±2.3	0.7±1.5	0.9±0.6	0.6±0.7
	(0[e])	(90[a])	(95[a])	(94[a])	(96[a])
Mean reduction	0	85.7	85.7	88.3	81.7

() Reduction percentage in seed to seedling transmission based on recovery from seedlings

[a]: Values in the same row followed by different letter are significantly different (P<0.05). Data are Means±SD of six, five and four replicates for *A. padwickii*, *B. oryzae* and *F. moniliforme*, respectively. Each replicate is average of four recordings from two separate experiments

respectively. These reductions were significantly ($P<0.05$) higher than the 64% reduction obtained with Dithane M-45 (Table 2, Fig.4).

Despite complete suppression of *B. oryzae* on seeds treated with EOs of *T. vulgaris* and *O. gratissimum*, close to 2 % seedlings raised from these seeds were infected compared to 11% of seedlings from untreated seeds (Table 2).

Control of Seed-borne Infection and Seed to Seedling Transmission of *F. moniliforme*: The Eos from *C. citratus*, *O. gratissimum* and *T. vulgaris* were equally and highly effective against *F. moniliforme* and just as effective as Dithane M-45, in controlling the seed infection by 95-100 %. The recorded differences among the EOs and Dithane M-45 were not statistically significant however their effect was significant ($P<0.05$) compared to the untreated control (Table 1). The treatments with the three EOs and Dithane M-45 reduced significantly the seed to seedling transmission of *F. moniliforme*. Less than 1% (0,7 - 1%) seedling infection was recorded from treated seeds compared to 15% from the non-treated control (Table 2). There were no significant differences ($P>0.05$) between the reduction of the seed to seedling transmission of *F. moniliforme* obtained with Dithane M-45 and the EOs. The level of *F. moniliforme* infected seedlings exceeded by a factor 2, 5 the level of seed infection in the non-treated control and a higher incidence of infection of seedlings than corresponding seeds was also recorded for the *C. citratus* EO treated seeds (Table 1 and 2). Infections were recorded in seedlings raised from four out of the five seed samples infected by *F. moniliforme*.

Improvement of the Germination of Seeds Treated with Essential Oils and Dithane M-45: The treatments with the Eos increased the level of germination from 61% as means for 7 varieties of non-treated seeds to 64, 66 and 69% for treatments with *C. citratus*, *T. vulgaris* and *O. gratissimum*, respectively and 73% for Dithane M-45 treated seeds. Germination of EO-treated seeds was ranging from 46% to 85% among the seven cultivars, compared to the 26 to 74% recorded from the non-treated controls (data not shown).

Higher and statistically significant differences were found between the improvement of germination induced by the treatment with Dithane M-45 and EO from *O. gratissimum*, compared to EOs from *C. citratus* and *T. vulgaris* (Fig. 2). In addition, rice plants raised from the treated seeds appeared greener and stronger (Fig. 3, 4).

DISCUSSION

Results of our current study indicate that each of the three EOs tested, reduced the natural infection frequency of *A. padwickii*, *B. oryzae* and *F. moniliforme* in rice seeds. These fungi which are well-known important seed-borne pathogens of rice were found in seed samples of the seven cultivars tested with mean infection level varying from 6% to 34% (Table 1). Treatment effect was statistically significant ($P<0.05$) compared to the non-treated control .

The EOs from *O. gratissimum* and *T. vulgaris* displayed the same high effect as Dithane M-45 towards *A. padwickii*, *B. oryzae* and *F. moniliforme* reducing the seed infection by 95% to 100% (Table 1). Such a strong effect has previously been obtained with EO from *O. gratissimum* towards *F. moniliforme* in maize seeds reported by Tagne *et al*. [20, 24]. The EO from *C. citratus* was in general slightly weaker than the two other EOs but more effective against *B. oryzae* and *F. moniliforme* than against *A. padwickii* which was only reduced by 48%. These results indicate that the EOs tested have the potential as control agents of the seed-borne fungi studied. The similarity between the antifungal activity of EOs from *T. vulgaris* and *O. gratissimum* and their superiority to *C. citratus* could be attributed to their contents of known antimicrobial compounds. Such chemical compositions have been studied by Amvam Zollo *et al*. [15] and Nguefack *et al*. [18] who found high content of thymol (46.2 and 27.2%) followed by γ-terpinene (20.0 and 22.7%), *p*-cymene (7.0 and 23.6%), carvacrol (0.2 and 3.3%) and linalool (0.4 and 5.2%), respectively, for the Eos from *O. gratissimum* and *T. vulgaris*. Nguefack *et al*. [18] have also demonstrated that the EO from *C. citratus* contained mostly citral a and b. Thus the similarity between the control effect of EOs from *O. gratissimum* and *T. vulgaris* could be correlated to the similarity of their chemical composition. A similar conclusion was made by Tassou *et al*. [30] who studied the mode of action of EOs and of their phenolics. They reported that the terpenes containing alcoholic compounds are more effective against micro-organisms than the ones having aldehydic function. This could explain the lesser control effect recorded with the EO from *C. citratus* which contained mainly aldehydic terpenes as compared to those from *O. gratissimum* and *T. vulgaris* which are rich in terpenic compounds with alcoholic function. In most cases the recovery of the fungi in the seedlings was slightly higher compared to the level of infection recorded in blotter tests of treated and untreated

Fig. 1: Non-treated rice seeds covered with greyish black fluffy mycelium of *B. oryzae* (centre) and cleaner seeds after treatments with Dithane M-45 (bottom left) or with essential oils from *T. vulgaris* (bottom right), *C. citratus* (top left), and from *O. gratissimum* (top right)

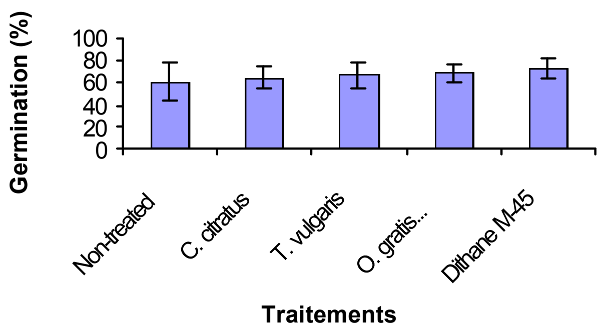

Fig. 2: Effect of seed treatments with EOs and Dithane M-45 on germination (Mean germination ± SD of seven rice cultivars)

Fig. 3: Rice seedlings (14-day-old) emerged from: Non-treated seeds showing "bakanae" disease caused by *F. moniliforme* (right); treated seeds with the EO from *T. vulgaris* (left) showing healthier and greener seedlings

Fig. 4: Portions of rice seedlings (30-day-old) emerged from: non-treated seeds (right) showing black and pink discoloration of blotter by *B. oryzaee* and *A. padwickii*, respectively; treated with Dithane M-45 (centre) and with EO from *T. vulgaris* (left) with healthier seedling portions

seeds but it came as a surprise that as much as 2-3 times more seedlings were infected by *F. moniliforme* than revealed by the blotter method. These are probably deep-seated infections or resting mycelium according to Fazli and Schreder [31], Ou [32] and Vidhyasekaran *et al*. [33]. However the control of the deep-seated *F. moniliforme* infections recorded (Table 1 and 2) indicates very clearly the ability of EOs to migrate into the rice seed. As a consequence the three EOs also reduced the seed to seedling transmission of *A. padwickii*, *B. oryzae* and *F. moniliforme* (Table 2). The fungi could be recovered from 11 to 18% of seedlings raised from non-treated seeds and from 1-4% of seedlings raised from seeds treated with the respective EOs and from Dithane M-45 treated seed. Improvements of the germination ranging from 5-13% were recorded in all the seven cultivars tested after treatments with the EOs (Fig. 2). This improvement was about 8% to 13% with the EOs from *T. vulgaris* and *O. gratissimum* and 5% for *C. citratus*. This is to our knowledge the first report on EOs as germination stimulants of rice seeds. The lower effect on germination for EO from *C. citratus* could be related to its high content of citral (81%) [18], which are oxygenated monoterpenes known to have negative effect on germination of soybean seeds[34].

In this present study it is well demonstrated that the control effect of Eos from *C. citratus*, *T. vulgaris* and *O. gratissimum* on the three most important seed-borne fungi of rice (*A. padwickii*, *B. oryzae* and *F. moniliforme*) is well demonstrated and that the EOs could be used as seed treatments for rice with the same expected output as obtained with Dithane M-45. It is therefore concluded that the three EOs tested are potential bio-fungicides against the major seed-borne pathogens of rice and as such contributed to characterise these EOs as "broad spectrum" bio-fungicidal chemicals. Paveley and Davies [35] have encouraged the use of such chemicals as seed treatments. The different properties of the three EOs demonstrated in this work showed that they also satisfy most of the demands for a bioactive chemical for seed treatment given by Hewett and Griffiths [36] and Schwinn [37] who stated that it should protect against both seed- and soil-borne pathogens, control deep-seated seed-borne pathogens and stimulate germination and/or enhance growth during the seedling stage.

Essential oils for seed treatments may become a "panacea" for rice crop protection. However, further investigations and product developments are needed as well as field experiments which are ongoing in Cameroon for the third year.

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