Response of Cowpea (*Vigna unguiculata* L. Walp.) Seeds in Culture to Different Rates of Seed-Treatment with Phytochemicals from *Piper guineense* L.

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ABSTRACT
Seed-borne mycoflora can severely affect seed viability and germination, seedling vigour, crop performance, quality and yield. Seed treatments present a cheap and veritable means for their control. However, owing to ecological and mammalian toxicities, alternatives to synthetic pesticides are being sought. The aim of this study was to assess in vitro *the effects of 7 rates (0, 5, 10, 15 20 25 and 30%) of* Piper guineense *L. as seed-dressing phytochemicals on the germination of the treated cowpea seeds and the incidence of the seed-borne fungi and to determine the rate(s) that is most fungitoxic to the seed-borne mycobiota, and least phytotoxic to the cowpea seed. The experiment was laid out in completely randomized design (CRD), with 4 replicates; and was repeated twice. The results showed that the germination profile of the treated seeds deceased with increasing dose of application of the phytochemicals; with best germination of 80% obtained at 5% concentration. This study also affirmed that the mycobiota* Fusarium oxysporium, F. verticilloides, Colletotrichum destructivum, C. truncatum, Aspergillus fumigatus, A. flavus, A. niger and Rhizopus spp. *were associated with seeds of cowpea variety IAR-48. It indicated that though 30% of the phytochemicals significantly (P≤0.05) gave a better reduction of these seed-borne fungi; however, germination was better at 5% dose of application. This was followed by 10%, 15% etc. Hence, 5-15% aqueous root extracts of* Piper guineense *L. could be used as effective seed-dressing phytochemical in Integrated Disease Management (IDM) Programmes for the control of seed-borne fungi of cowpea, for improved productivity. However,* P. guineense *seed extract treatments should be tried out in further studies to determine the best length of exposure (soaking period) to the treament.*

Key Words*: Seed-borne fungi, Phytochemicals, Germination, Piper guineense L., Concentration*

INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp) is one of the most important grain legumes in the world especially in Sub-Saharan Africa (SSA) and Asia where it provides essential nutrients and

proteins to 110 million consumers of the crop (Obi and Barriusa-Vargas, 2013; Awurum et al., 2013). In farming systems of such localities, the crop also play roles in improving the soil nitrogen status, control erosion and suppress weeds (Awurum et al., 2001; 2014). One hundred grams (100g) of its grains amongst other vital nutrients is reported to contain about 23% protein and 56% carbohydrate while same quantity of the leaves are 9% protein-rich (Infonet-Biovision, 2014). This high nutrient status endears its use in several tropical food forms, pot-herbs and livestock fodder.

However, cowpeas are susceptible to attacks by several fungal organisms at all stages of their growth (Ajibade and Amusa, 1994; Amusa et al., 2001; Enyiukwu and Awurum, 2013). Some of these organisms are seed-borne and seed transmitted. Many workers have reported the association of *Fusarium oxysporium F. equiseti, F. verticiloides, Aspergillus niger, A. flavus, Penicilium digitatum P. crysogenum, Rhizopus arrhizopus,* and *Rhizotonia solani* with seeds of the crop (Kritzinger, 2002; Mogle and Maske, 2012; Makun et al., 2012). Seed-borne fungal diseases can severely affect crop yield and quality. They reduce seed viability and germination, and contribute to poor seedling vigour. These lead ultimately to poor crop performance and reduced yield (Van der Wolf et al., 2007; Akinbode and Ikotun, 2008).

Besides good agronomic practices, control of these mycoflora has largely been done by seed treatments and conventional foliar sprays with synthetic fungicides. However, Van der Wolf et al. (2007) asserted that seed treatment is preferred over field sprays. Reasons being that relatively low amounts of active ingredients are required for seed treatment; and that applications can be done in contained areas. Thus, reducing associated health risks and drift-borne hazards from the crop protection sprays. In a study, Makun et al. (2012) found Apron Star a synthetic fungicide to reduce the seed-borne fungi associated with cowpea seeds by 93%. This efficacy notwithstanding, in recent times synthetic fungicides are being de-emphasized due to development of resistance to effective fungicides by pathogens and pesticide residues in treated crops. As a result alternatives are being sought from plant sources (Asawalam and Adesanya, 2001; Awurum et al., 2005). An evaluation indicated that Biosept (33% grapefruit extract) impeded the development of fungi on some vegetables as effectively as a synthetic fungicide Sarfun T65DS (Szopinska et al., 2007). So also did extracts of *Diodia scandens* SW and *Cyathula prostata* L. on seed-borne *Colletotrichum spp.* of cowpea (Ogu and Iwoye, 2013)

We have previously reported the potency of *Piper guineense* L. extracts as seed-dressing phytochemicals against seed-borne fungi of cowpea (Enyiukwu and Awurum, 2011; 2012; Awurum et al., 2014). And have also reported the toxicity of the extract applications to the seed embryo; at the considered concentrations (Enyiukwu and Awurum, 2013b). Therefore, this present study was undertaken to assess the different rates of phytochemicals from this spice plant (*Piper guineense* L) to find its seed-safe level(s) that can be used in low-input agriculture for cowpea seed health management against fungal mycoflora.

MATERIALS AND METHODS

Sources of seeds and plant materials

The experiment was conducted in the Crop Science laboratory of Michael Okpara University of Agriculture, Umudike. The seeds of cowpea variety IAR-48 sourced from the Research and Training (R&T) Unit of the University; and dried seeds of *Piper guineense* obtained from Umuahia main market, were used in the study.

Preparation of plant extracts and culture medium

Dried seeds of *Piper* guineense L. were air-dried on the laboratory bench at room temperature of 27^{o} C overnight. About 150g of the seeds were weighed out and ground into powder, using an electric blender (Model: XL 15). Thereafter 5g, 10g, 15g, 20g, 25g and 30g of the powdered specimen (*Piper guineense* L. seeds) were weighed out separately. Each powder was put into a 200mL beaker to which 100mL of sterile distilled water was added and allowed to stand for 1h. At the end of the hour, the pastes were strained through 4-folds of sterile cheese cloth, to obtain filtrates of corresponding strengths of the plant materials. Then 200g of fresh peeled Irish potato was boiled in 1L of water contained in 2L flask for 1h. The broth was filtered through double-folds of sterile cheese cloth, made up to 1L with sterile distilled water to which 20g agar and 15g glucose were added. The broth was modified with 20mg of gentamicin (an antibiotic to kill or suppress any bacteria contaminating the medium) and stirred vigorously with a glass stirrer. The flask was stoppered with foiled cotton wool and then autoclaved at 120^{o}C and 152cmHg for 30 minutes.

In vivo experiment

Forty (40) cowpea seeds (Var.IAR-48) were soaked in each aqueous suspension of the respective strengths (5, 10, 15, 20, 25 and 30%) of the filtrates of the plant materials for 2 h, removed and air-dried at room temperature (27^{o}C) for 1 h. The control was set up in a similar manner but consisted of cowpea seeds soaked in sterile distilled water for the same length of time. About 20mL of PDA was poured aseptically into each Petri dish in the inoculation chamber and allowed to solidify for 2 h. At the end of this period, 10 seeds soaked in a particular strength of plant extract were then plated in the Petri dishes and incubated for 5 days at 27^{o}C, and then observed for germination and mycelial growth. Ten (10) cowpea seeds were plated per Petri dish per replicate. The whole experiment was repeated twice giving 40 seeds per treatment and 280 seeds for each set of observational units. The Petri dishes were arranged in a completely randomized design (CRD) in the inoculation chamber. At the end of 5 days, counts of germinated seeds were taken per plate and means of replicates recorded. Records of the seed-borne fungi that grew out of the seeds and sporulated were also taken.

Pure cultures of the mycoflora associated with the cowpea seeds were obtained by repeated sub-culturing of the organisms on solidified 20mL PDA in the Petri dishes. Using a sterile needle, bits of each seed-borne fungus were transferred on glass slides, stained with a drop of lactophenol in cotton blue, teased gently and covered with cover slips. The slides were fixed by gently passing them over a spirit lamp flame, then mounted on the stage of a microscope, observed and the various mycoflora identified through their spore characteristics by the aid of fungi identification manual by Banett and Hunter (1995),, illustrated genera of *Colletotrichum* species by Damn et al. (2009; 2014) and monographs of the International Mycological Institute IMI (1995).

The percentage incidence of the seed-borne fungi on the incubated cowpea was assessed based on the the formular by Amadioha (2003) as:

% Incidence = Number of seeds infected/Total number of seeds examined x 100/1 The percentage germination of the incubated cowpea seeds was also assessed as:

% Germination = Number of infected seeds/Total number of seeds examined x 100/1

STATISTICAL ANALYSIS

All measurements collected from this study were 4 replicates and were analyzed by simple percentages and analysis of variance (ANOVA) using Genstat Release (PC/Windows Vista version 12.10) at 5% level of significance. Fisher's least significant difference (FLSD) at $P \leq 0.05$ was applied to assess the differences amongst the means.

RESULTS

The result showed that the germination profile of the treated seeds in general, increases with decreasing concentration of the test extract Table 1. The best germination of 80% was obtained at 5% strength of application while 30% concentration gave the least germination of 58%. The seed-borne mycoflora found associated with the cowpea seeds in this investigation were *Aspergillus fumigatus, A. flavus, A. niger, Colletotrichum destructivum, C. truncatum, Fusarium oxysporium, F. verticilloides* and *Rhizopus sp*. The profile of the fungal load on the treated cowpea seeds, also revealed that the seed-borne mycoflora of the treated cowpea seeds were decreasing with increasing strength of application of the test extracts. It showed that 5% concentration of the test extract reduced the fungal load from 49.10% on the untreated seeds to 7.94 Table 1.

Table 1: Effects of the phytochemicals from *P. guineense L.* on the germination and seed-borne mycoflora of the dressed cowpea seeds.

Strength of Plant material (%)	Mean Disease Incidence (%)	Mean seed germination (%)
Piper guineense L. 5%	7.93	80.09
Piper guineense L. 10%	7. 61	78.03
Piper guineense L. 15%	7.45	75.23
Piper guineense L. 20%	7.03	69.71
Piper guineense L. 25%	5.82	67.22
Piper guineense L. 30%	5.03	58.09
Piper guineense L. 0%	48.60	42.93
LSD	**0.30**	**0.18**

DISCUSSION

Findings from this present study showed that fungi of the genera *Colletotrichum, Fusarium, Aspergillus,* and *Rhzopus* were associated with cowpea seeds (Var. IAR-48). This agreed with previous reports from Makun et al. (2012) and Mogle (2013) who also found mycoflora from these genera associated with seeds of cowpea. It also upheld our earlier reports of these genera of seed-borne fungi in this variety of cowpea. However, in this present finding, *Curvularia* species were absent (Enyiukwu and Awurum, 2013). The absence of *Curvularia sp*. may be due to changes in on-farm environmental conditions and/or cropping system between the previous study years and now. This may have disadvantaged the growth and development of the fungus on the crop.

The increase in germination with decreasing dosage of the test extract suggests that high doses of the treatment are toxic to the cowpea seed embryo. This thus, upholds our previous finding where seeds exposed to 50% strength of the phytochemical failed to germinate completely. A similar study by Van der Wolf (2007) with essential oils on seed-borne bacteria of vegetables,

also demonstrated that higher concentrations of the test oils had profound negative effects on the seed germination; further supporting our present findings.

The toxity of some plant derived phytochemicals to seed-borne fungi in legumes have been reported. Several storage moulds (*Penicilium crysogenum, F, oxysporium, F. equiseti and A. flavus*) for instance, were severely impeded by Thyme oil and Biosept (Kritzenger, 2002). Nwachukwu and Umechuruba (2001), found phytochemical from *Carica papaya, Venonia amygdalina, and Azadirachta indica* effective in reducing the fungal load on African yam bean (*Shenostylis stenocarpa* L.) seeds. Akinbode and Ikotun (2008) showed that *Moringa oleifera* leaf extracts inhibited seed-borne *Colletotrichum destructivum* in cowpea; while phytochemical from *Xylopia aethiopica* and *A. indica* retarded both colony growth and spore germination of the same fungus (Obi and Barriusa-Vargas, 2013). *Sclerotium rolfsii* cause of basal stem rot of cowpea; and *Fusarium oxysporium* which attacks Okra plant was found sensitive in similar evaluations to phytochemicals from *Afromonium meleguata, Monodora myristica* and *Citrus spp.* (Okwu et al., 2007; Okwu and Njoku, 2009). These retardations were found to follow a dose dependent manner (Mogle and Maske 2012). Our present findings are congruent with these submissions. The increasing toxicity of the *Piper*-derived extract on the seed-borne fungal organisms connotes however, that the presence of the active ingredient(s) of the plant material was increasing in the aqueous medium; and hence was better able to enforce the observed fungitoxity.

Seed treatment is seen as a favoured technology over field sprays. It involves relatively low amounts of active ingredient; and applications could be done in contained areas. Thus, reducing the associated health risks and drifts from crop protection sprays (Van der Wolf et al., 2007). Therefore, in conclusion, the findings from this work support the use of 5- 15% strength of *Piper guineense* L. for dressing cowpea seeds against a wide range of seed-borne fungal diseases.

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