DEVELOPING A ONE-STOP CULTURAL CHARACTERIZATION FOR THE DETECTION OF MYCOTOXINS-PRODUCING FUNGI IN MAIZE ANND COWPEA

Lasisi, S. A., Bada, B. S., Anjorin, T. S., Shittu, O.B. and Olatunde, K.A.
[1]*Department of Environmental Resources Management and Toxicology, College of Environmental Resources Management, Federal University of Agriculture, Abeokuta, Nigeria*
[2]*Department of Microbiology, College of Biological Sciences, Federal University of Agriculture, Abeokuta, Nigeria*
[3]Department of Crop Protection, Faculty of Agriculture, University of Abuja, Nigeria

Correspondence:lasisi_abidemi@yahoo.com **Telephone:** +2347019332627

ABSTRACT
The global cost and health effects of mycotoxins contamination puts a lot of pressure on the need to urgently develop cheaper and reliable presumptive alternatives for the detection of mycotoxins in foods. This study attempted to develop a one-stop cultural characterization for the detection of mycotoxins-producing fungi in cowpea and maize grains. A comparative analysis of the structural chemistry of surfactin obtained from the supplied FTIR spectra and the standard was carried out. Thermodynamic stress tests are done to determine freeze-thaw cycles and low and high-temperature stability. Phyto-toxicity rating, distribution coefficient, soil organic carbon coefficient, and 14-day stability test, were also assessed. The capacity of the formulation for cultural characterization of mycotoxins-producing fungi was evaluated. In the FTIR spectra, peaks observed at 1651.5cm-[1] and 3479.90cm-[1] for –C=C-and N-H stretch vibrations, respectively confirmed the presence of aliphatic primary amide, indicating peptide, a surfactin. The test formulation at 3.2 ml/0.12L showed no observable effect(s) or symptomatic sign, except for stunting with a phytotoxicity rating of 1 in maize grain. The formulation and modified sample showed Kd and Koc values of 2.34 and 4.9 respectively. The decomposition rates measured for both the test and modified formulation based on electrical conductivity were 2.17 and 1.23% respectively. Isolates on Sabouraud Dextrose Agar used for presumptive test for identification of mycotoxins- producing fungi showed an average of 25, 13, and 5 colonies for TFP (Test Formulation Biopesticide); SCP (Synthetic Chemical Pesticide) and TM (Traditional Method) after 12 months storage. No colony of any fungus was formed in CN (Negative Control) due to the accumulation of toxic secondary metabolites from fungal growth (Plate 1). Developing of a rapid and sensitive presumptive protocol for qualitative detection of mycotoxins devoid of clean-up process associated with the conventional detection methods.

INTRODUCTION
Mycotoxins are toxic secondary metabolites produced by fungal species that infest agricultural commodities either on the field or under suboptimal conditions and make them unsafe for human and animal consumption. These agricultural commodities include wheat, cowpea, corn, spices, wine and cocoa, coffee, and dairy products via secondary exposure routes (feeds). The consumption of commodities contaminated with mycotoxins, cause severe effects in human and animal health (Zheng, *et al.,* 2006; Rodrigues *et al.,* 2011). These include mutagenic, nephrotoxicity, teratogenicity, and carcinogenic (Chauhan, *et al.,* 2016).

It is estimated that due to the harmful effect of aflatoxins, approximately 25% of agricultural products get damaged worldwide (Yard, 2013). The global economic cost due to mycotoxins is estimated to run into billions of US dollars (Swamy *et al.,* 2004). This puts a lot of pressure

on mycotoxin risk management concerning the type of mycotoxins; sampling procedure to adopt and when; where; and number of tests to conduct. An appropriate and accurate sampling procedure is also a very crucial step in analyzing mycotoxins because an inappropriate sampling can lead to false results, causing devastating health and economic risks.

Microbiological quality and safety of food products have sustained interest in the use of mathematical models for quantifying and predicting microbial behaviour (Lahlali *et al.,* 2005). According to Garcia *et al.* (2009), the predictive model for filamentous fungal growth has not received the same attention as bacterial growth. Bacteria reproduce by fission and would normally grow homogeneously in a liquid medium or take place only on surfaces, while fungal hyphae penetrate the physical three-dimensional matrix of food (Dantigny *et al.,* 2005). Measurement of hyphal extension rate, generally reported as radial growth rate in mm/h or mm/d, is probably the most often used measurement, but it does not represent the true tridimensional fungal growth. According to Garcia *et al*. (2009), a linear increase in colony diameter over time occurs in fungal growth.

Colony diameter is the most simple and direct form to measure mould growth (Gibson and Hocking, 1997). According to Garcia *et al*. (2009), a linear increase in colony diameter over time occurs in fungal growth.

A rapid and cheaper alternative for the presumptive evaluation of farm produce and feed for mycotoxin contamination is urgently needed. The current conventional analytical methods, aside, from the protection of the food supply system; through effective mycotoxin risk management, offer a platform for the generation of reliable food safety data for both local and international trade. However, these methods for mycotoxin analysis frequently relied on purified extraction, which involves extract clean-up and separation and produced results slightly higher than the limit set by the European Committee on Standards (CEN). For example, the Fluorometric method has a limit of 5-5000 ppb for aflatoxin, whereas the European limit is slightly lower (4 µg kg^{-1}). According to Goodman and Scott (1989), mycotoxins ranked higher than the important synthetic chemicals, contaminants, plant toxins, and pesticide residues as the most important chronic dietary risk factor.

Research into biosafety of some traditional bio-control measures to determine their efficacy and toxicity to man, animal, and crop plants, has received adequate attention in many literature (Dimetry, 2014). Traditionally, various herbs and spices such as turmeric, garlic, and cloves, are used by people for the management of storage pests (Dimetry, 2014). These herbs possess antioxidant capacity, due to the presence of phenolic compounds, which enable them to scavenge free radicals, chelate transition metals, and enhance the activities of antioxidant enzymes (Rubio *et al*., 2013).

This current study aimed at developing a simple one-stop presumptive protocol for the detection of mycotoxins-producing fungi in maize and cowpeas.

Materials and Methods

The formulation, which was a surfactin-saponin-based bio-pesticide from a research group on **SAFE PESTICIDES USE** at the Department of Environmental Resource Management, Federal University of Agriculture, Abeokuta, with data on chemical identity using FTIR Nicolet Is50 Spectrometer available at the Africa University of Science and Technology, Abuja. Results obtained from the characterization of the active ingredient were also made available.

Determination of biopesticide's quality characteristics

Parameters such as coefficient of distribution, electrical conductivity, and pH as a function of stability test, were all carried out at the Soil Science Department's Laboratory, University of Abuja. Thermodynamic stress tests: freeze-thaw cycles, low and high-temperature tests carried out using the methods described by Shao *et al*. (2018) with slight modifications. The test formulation was stored at -18^0 C for 48h and

thereafter, observed for any change of state or floating of oil and appearance. After thawing at 27 ^{0}C the test sample was left to stand for another 48h and observed for any floating oil and appearance as before.

Cloud-Point: The method described by Shao *et al.*, (2018) with slight modification was also employed. The solubility of the test sample at the pasteurization temperature (65^0 C) was determined by taking 10 ml of the test sample and centrifuging it at 500 rpm for 15 minutes, which was observed for any phase separation that determined physical stability.

Electrical conductivity: Dist Conductivity/ DS meter was used for the electrical conductivity test of the biopesticide and an improved test sample after a natural UV-protector and natural stabilizer were added.

Phytotoxicity rating of test bio-pesticide

The crops were examined for the presence of phytotoxicity effects. The type and extent of these effects were based on the protocol described in FAO/AP/27. The test bio-pesticide formulation was applied at a concentration of 3.2 ml/0.12l.

Screening of mycotoxins-producing fungi

About fifty kernels of grains of each sample lot were assayed by indirect plating technique for internal mould infection as described by Pitt *et al*.(1997) and Tibagonzeka *et al*. (2018), with some modifications used for mycotoxins producing screening. The kernels used for mycotoxins-producing fungi screening were surface disinfected for 3 minutes with sodium hypochlorite (10%), washed three times with sterile distilled water, and air dried. The kernels were milled into flour using a blender and were later filtered. A 9:1 dilution was prepared by suspending 10 ml of the sample in 90 ml of sterile water. In each case, one milliliter of initial suspension was transferred in succession into 9 ml of diluent to make dilutions of 10^{-2} to 10^{-5}. About 0.1 ml of the selected dilutions were surface spread onto the Sabouraud Dextrose Agar using a 90˚ glass rod. All the samples were plated in duplicates. The plates were incubated upright at about 25˚C for 5 days.

The colonies formed on the plates were sub-cultured on SDA for five days to purify and determine secondary character such as the growth rate useful in a predictive model for the growth of mycotoxins-producing fungi.

Statistical analysis

Analysis of variance (ANOVA) was done to determine the significance of results from insect prevalence level, severity of disease, and effect of test-biopesticide on storage data sets.

Results

A comparative analysis of the structural chemistry of surfactin obtained from the supplied FTIR spectra and the standard as shown in (Figures 1 and 2) reveals the presence of primary amide as –C=C- stretch vibrations, which is usually observed in the region 1680-1640 cm^{-1}. The N-H stretching vibrations observed at 3479.90 cm^{-1} confirm the compound to be an aliphatic primary amide. The N-H stretching vibrations for aliphatic primary amides are generally observed between 3500-3100 cm^{-1} with two bands due to asymmetric and symmetric stretching modes. Intermolecular and intra-molecular hydrogen bonding broadens the absorptions and lowers the frequency. The C-N stretching vibration of the aliphatic primary amide appeared in the fingerprint region at 1025cm $^{-1}$ and the N-H bending vibration for the amide was observed at 632.40 cm^{-1} shown in Figure 1.

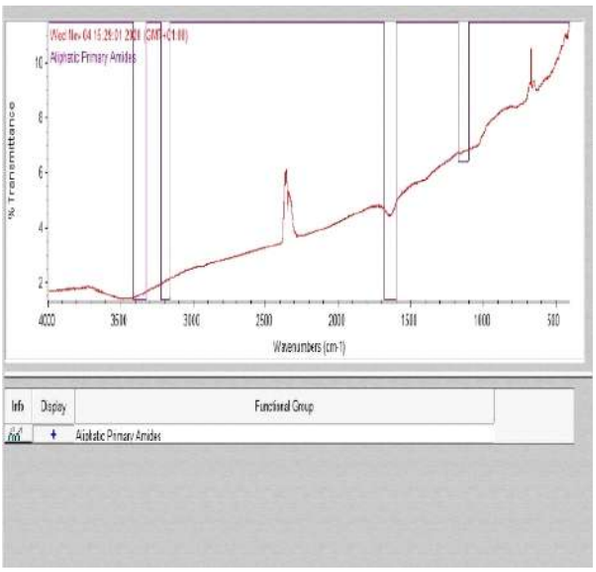

Figure 1: FTIR Spectra of Surfactin produced from *Bacillus subtilis*

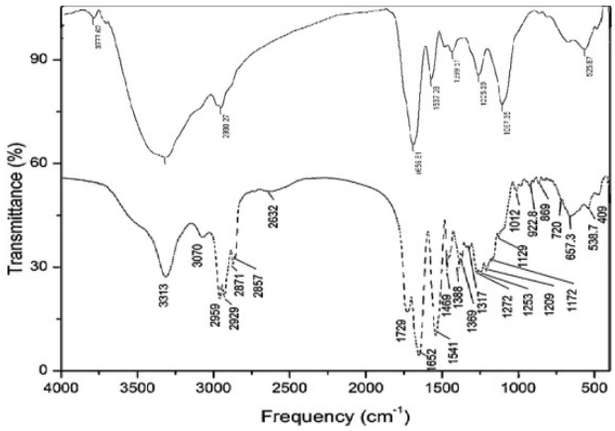

Figure 2: FTIR spectra for standard surfactin from Sigma-Aldrich, Germany, at the bottom, and the spectra for *Bacillus liquefeous* at the top for comparison with Figure 1. Source: El-Sheshtawy *et al.,* 2015

Distribution coefficient, K_d; Koc; pH and stability test

The results of the K_d, Koc, and pH after 14 days of stability test are shown in Table 1. Electrical conductivities of 8.5 dS/m and 8.7 dS/m were obtained as electrical conductivities for the test formulation and modified sample, respectively. The test formulation and modified sample showed K_d, and Koc values of 2.34 and 4.9, respectively, and differed in the mean pH value with 4.3 recorded for the modified test sample, which makes it more acidic. The modified sample was introduced to see the effect of the sun-screening effect of soya beans and water-soluble curcumin on the reduction of photooxidation.

The solubility of both samples at a temperature above the pasteurization temperature (65 ^{o}C) decreased with a more turbid appearance as shown in Table 1. The cloud point of a bio-pesticide formulation reported by Shao *et al*. (2018) was above 72 ^{0}C. The low-temperature storage stability of the test sample revealed transparent and uniform preparation and no phase separation.

The decomposition rates measured for both test biopesticide and modified samples based on electrical conductivity were 2.1 and 1.2 %, respectively. The test biopesticide had a higher electrical conductivity value and higher decomposition rate, hence less stable than the modified sample. The decomposition rate Shao *et al*. (2018) obtained for a bio-pesticide was 2.1%. Sunlight, rain, temperature, and humidity all determine the rate of degradation of microbial products once they are applied to foliage in the field. The UV-A and UV-B components of sunlight are major contributors to the degradation of biopesticide and are largely responsible for short field persistence of biopesticide (Jaronski, 2009). Increasing photostability of bio-pesticides by using natural plant extracts (Eyheraguibel *et al*., 2010), soy-based sunscreens, and water-soluble lignins as adjuvants for UV protection (Behle *et al.*, 2011) have been demonstrated.

Table 1: Biopesticide mobility and stability parameters in the soil

Pesticide Type	K_d value	Koc value	pH 24h	pH 7 days	pH 14 days
TFP	2.34	4.9	5.97	5.64	5.10
SCP	9.37	19.60	5.68	5.68	5.68
MTFP	2.34	4.9	4.30	4.13	4.01

TFP = test biopesticide formulation; SCP = Synthetic Chemical (Lamda Cyhalothrin); and MTFP = Modified testbiopesticide formulation

Table 2: Stability tests of test-biopesticide over 14 days' storage

Pesticide Type	Stability (EC dS/m) 24H	7days	14days	Low temp. Appearance	High temp. Appearance	Cloud- Point (^{0} C)
TFP	4.01	5.48	5.76	Translucent	Slightly turbid	>65
SCP	-	-	-	Transparent	Transparent	>
MTFP	4.36	4.66	5.27	Translucent	Slightly turbid	>65

TFP = test biopesticide formulation; SCP = Synthetic Chemical (Lamda Cyhalothrin); and MTFP = Modified test biopesticide formulation

Phytotoxicity rating of test bio-pesticide

The crops were examined for the presence or absence of phytotoxicity effects. The type and extent of these effects were based on the protocol described in FAO/AP/27. The test bio-pesticide formulation applied at a concentration of 3.2 ml/0.12l showed no observable effect(s) or symptomatic sign, except for stunting with a phytotoxicity rating of 1 for both TFP1 and TFP2 in maize crops. Table 3 depicts the result of the phytotoxicity rating for maize and cowpea.

Table 3: Phytotoxicity Rating of test bio-pesticide applied at 3.2 ml per plot

Treatment	Crop Response/injury* Yellowing	Stunting	Necrosis	Epinasty	Hyponasty
TFP1	0 (0)**	1 (0)	0 (0)	0 (0)	0 (0)
TFP2	0(0)	1(0)	0 (0)	0 (0)	0 (0)
SCP	0(0)	0 (0)	0 (0)	0 (0)	0 (0)

*Parameters stipulated in phytotoxicity assessment protocol FAO/AP/027 **Values in parenthesis are the phytotoxicity rating for cowpea.

Efficacy in Mycotoxins Reduction in Stored Maize and Cowpea

The isolates on Sabouraud Dextrose Agar used for the presumptive identification of mycotoxins-producing fungi showed an average of 25, 13, and 5 colonies for TFP; SCP, and TM after 12 months' storage. No colony of any fungus was formed on CN due to the accumulation of toxic secondary metabolites from fungal growth (Plate 1). According to Summerell *et al.* (2003), colonies of mycotoxins-producing fungi, growing on a medium for more than 2 or 3 weeks usually die, due to the accumulation of toxic metabolites, which are byproducts of the fungal growth in the medium. To avoid mixtures of morphological

characters, colonies formed on TFP, SCP, and TM were sub-cultured on SDA in triplicate for purity and secondary cultural characteristics, only TM showed radial growth with an average of 8.9 x 10^{-2} mm h^{-1} as shown in Plate 2. Colonies from TFP and SCP produced an average vertical growth rate of 1.3 x 10^{-1} and 1.49 x 10^{-1} mm h^{-1}, respectively (Plates 3 and 4). Plate 5 presents the culture morphology of TFP-treated samples on Chocolate agar, a Differentiation medium, which produced a creamy-grey colony with a rough and filamentous border made up of hyphae and pseudo-hyphae, within 48 hours of incubation. This simple method distinguishes *Candida albican* from other species of *Candida* as described by Sheth *et al*. (2005).

Plate 1: CN on SDA

Plate 2: TM sub-cultured on SDA

Plate 3: TFP sub-cultured on SDA

Plate 4 SCP sub-cultured on SDA

For developing countries such as Nigeria with limited capacity for mycotoxins monitoring and surveillance systems, a simple and reliable protocol for quick presumptive detection, is needed as no known processing method or cooking could reverse the mycotoxins status of food already contaminated. Elaborate sample clean-up processes associated with analytical methods are not needed for mycotoxin detection with this simple and natural novel method.

Plate 5: TFP Sub-cultured on Chocolate.Agar

Conclusion

The findings of this present work demonstrated the ability of the test formulation to prevent infection by mycotoxin-producing fungi, which makes it amenable for use as a reliable tool for developing a rapid and sensitive presumptive protocol for qualitative detection of mycotoxins, devoid of clean-up process associated with the conventional detection methods. The absence of any *Fusarium* or *Aspergillus* colony indicated the test formulation could control the secretion of secondary toxic metabolites in stored maize and cowpea. The *Candida* detected in the samples are normal commensal of the gastrointestinal tract.

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