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Full Length Research Paper

Effects of sound waves on conidiospores of *Aspergillus oryzae* strain RIB40 and characterization of the enzyme activity of rice–*koji*

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It was reported that the glucoamylase activity of rice-*koji* prepared with sound-irradiated conidiospores of yellow-*koji, Aspergillus oryzae,* at 16 kHz was decreased as compared with that of conidiospores that had not been sound irradiated. To reveal why the glucoamylase activity was changed, we observed the changes of the expression level of *glaB* encoding glucoamylase. We also determined to observe how the germination ratio of conidiospores and the hyphal weight of rice-*koji* were changed by sound waves. When conidiospores were irradiated with sound waves at 16 kHz, the expression level of *glaB* was decreased and the correlation between the expression level of *glaB* and glucoamylase activity was confirmed. When steamed rice was inoculated by sound-irradiated conidiospores, the conidial germination ratio 8 h after inoculation was 1.4 times higher than that of conidiospores that had not been sound irradiated, and the hyphal weight of rice-*koji* was 1.2 times higher than that of conidiospores that had not been sound irradiated. The correlation between the germination ratio and hyphal weight was also confirmed.

Key words: *Aspergillus oryzae* RIB40, glucoamylase activity, rice-*koji*; sound waves.

INTRODUCTION

In Asian countries, a microbial starter called *koji*, which is prepared with various microorganisms, is an important saccharifying agent in the production of fermented foods (Yamashita, 2021). *Aspergillus oryzae* and *Aspergillus sojae* are called *koji*- mold (Abe and Gomi, 2008), and *koji*-mold secretes large amounts of hydrolases, such as α-amylase and glucoamylase (Bennett, 2001). Rice-*koji* that *koji*-mold grown on steamed rice that secretes many enzymes from its hyphae, is widely used to make fermented foods unique to the region, such as Japanese sake and Chinese Shaoxingjiu (Nout and Aidoo, 2002).

The rice-*koji*-making process is affected by various environmental factors, such as light, temperature, and humidity (Okazaki et al., 1979). Among these environmental factors, the effects of sound waves on microorganisms have been highlighted in recent years. For example, it has been reported that when irradiated with low-frequency noise, bacteria representing *Staphylococcus* species exhibit resistance against antibiotic drugs, such as ampicillin (Kim, 2016). Furthermore, it has been reported that the growth of microorganisms involved in fermentation and brewing, such as *Saccharomyces* and *Lactobacillus* species, was enhanced by sound-wave radiation (Noguchi et al., 2011).

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In recent years, acoustic irradiation technology has been utilized in some breweries to improve quality. For example, it has been reported that the stimulation of the alcohol content of shochu is eased and aging is accelerated by irradiating with sound waves during periods of maturation (Noguchi et al., 2011). Despite the fact that the mechanism of the response to sound waves in *Aspergillus* species is unknown, it was reported that the glucoamylase activity of rice-*koji* was decreased as compared with that of rice-*koji* not irradiated when sound waves at 6.3 kHz were irradiated through the hyphal elongation stage of *koji*-mold 20 h after inoculation (Saigusa et al., 2015) (Figure 1B). Moreover, it was reported that the glucoamylase activity of rice-*koji* prepared with sound-irradiated conidiospores was not changed at 6.3 kHz and was suppressed 0.68 times at 16 kHz as compared with that of rice-*koji* prepared with conidiospores that had not been irradiated by sound (Matsumoto et al., 2021). These results suggested that the response to specific frequency sound waves was different between the stage of the hyphal elongation and the dormant stage of conidiospores. It was also suggested that the dormant stage of conidiospores of *A. oryzae* may respond to sound waves and may affect the enzyme activity of rice-*koji* prepared with sound-irradiated conidiospores. However, it is unclear how sound waves affect the growth of *koji*-mold, such as germination and hyphal elongation.

In this study, we determined to irradiate conidiospores with sound waves in the germination stage and observed how the germination ratio of conidiospores and the hyphal weight of rice-*koji* were changed by sound waves. Moreover, to reveal why the glucoamylase activity of rice-*koji* was changed by sound waves, we observed the changes of expression level of *glaB* encoding glucoamylase.

MATERIALS AND METHODS

Incubation of *koji*-mold on a solid medium

Yellow *koji*-mold, *A. oryzae* RIB40 NBRC100959, was purchased from the NITE Biological Resource Center (Tokyo, Japan). To suspend conidiospores, 1.0 mL of No. 707 liquid medium, described in the Data and Biological Resource Platform (https://www.nite.go.jp/nbrc/cultures/cultures/cultures.html), was prepared; the resultant aliquots were spread onto a potato dextrose agar solid medium (Nissui, Tokyo, Japan). The solid medium was sealed with surgical tape and incubated at 30°C for 5 days in incubator FS-620 (ADVANTEC, Tokyo, Japan).

Irradiation of conidiospores with sound waves and the preparation of rice-*koji*

Aliquots of 0.1 g of collected conidiospores were weighted exactly and added to a 50 ml Erlenmeyer flask with moistened filter paper at the bottom. Then the flask was covered with aluminium foil. The audio generator MINIRATOR MR2 (NTi, Tokyo, Japan) was used to generate sound waves. An earphone (YAZAWA Co. Ltd., Tokyo, Japan) was fixed to the head of the flask with vinyl tape. Conidiospores were irradiated by sound waves with 1.0, 6.3, and 16 kHz at 25°C for 24 h, and the power level was 5.0 dB (Figure 1A). For the control experiment, conidiospores incubated at 25°C in the absence of earphones, that is, not irradiated by sound waves, were prepared.

Aliquots of 100 g of commercial polished rice (*Oryza sativa* var. *Japonica* cv. *Hinohikari*) that was cultivated in Kumamoto Prefecture, Japan, were added to a 300-ml flask containing 30 ml of distilled water and left for 30 min. This aliquot was steamed at 105°C for 15 min using autoclave (BS-245, TOMY, Tokyo, Japan) to prepare steamed rice. After cooling to 40°C, 0.1 g sound-irradiated conidiospores were added to the flask. For the control experiment, 0.1 g conidiospores that had not been sound irradiated were added to the flask. The resulting mixtures were divided into two glass Petri dishes. Unless otherwise noted, rice-*koji* was made by incubating at 30°C for 36 h in a silence condition.

Extraction of enzymes from rice-*koji*

To extract the enzymes solution, 10 g of rice-*koji* was collected every 24, 36 after incubation, and was soaked in a mixture of 50 ml of 0.5% (w/v) sodium chloride solution containing a 0.2 M acetic acid buffer (pH 5.0) at 5°C for 18 h. The resulting extract was filtered and dialyzed with Visking tubing (5 nm, Nihon Medical Science, Osaka, Japan) against 1 L of a 0.01 M acetic acid buffer (pH 5.0) at 5°C for 24 h to remove the sugars or amino acid residue. This extract was used as an enzyme solution.

Determination of the hyphal weight of *A. oryzae* RIB40 cells in rice-*koji*

To reveal how the hyphal weight of rice-*koji* prepared with sound-irradiated conidiospores was changed, we measured the hyphal weight of rice-*koji*. Our procedures were carried out in accordance with the methods described in Masuda et al. (2009). To prepare extract containing enzymes, 2.0 g of ground dried rice-*koji* was weighed accurately, suspended with 10 ml of a 50 mM phosphate buffer (pH 7.0) and centrifuged at 2,270 × *g* for 10 min. The supernatant fluid was discarded. These processes were repeated four times to remove the ingredients. To degrade the cell wall of rice-*koji,* 10 mg of Yatalase (Takara Bio Inc., Kusatsu, Japan) was weighted accurately and added to the suspension; it was incubated at 37°C for 4 h with shaking. The resulting mixture was centrifuged at 2,270 × *g* for 10 min to collect the supernatant fluid, and 0.5 mL of the supernatant was added to 0.1 ml of a 0.8 M boric acid buffer and incubated in boiled water for 3 min. To cause a color reaction in N-acetyl-D-glucosamine, 3.0 mL of 10% (v/v) *p*-dimethyl-amino-benzaldehyde was added and incubated at 37°C for 20 min. Absorption was measured at 585 nm with spectrophotometer U-1800 (Hitachi High-Tech Inc., Tokyo, Japan).

Determination of the glucoamylase activity of rice-*koji*

To reveal how the glucoamylase activity of rice-*koji* prepared with sound-irradiated conidiospores was changed, the glucoamylase activity of rice-*koji* was measured according to the official methods described in Taniguchi (1993). For an enzymatic reaction, 0.1 ml of properly distilled extract solution was mixed with 1.0 ml of 2.0% (w/v) soluble starch solution (Nacalai Tesque, Kyoto, Japan) and incubated at 40°C for 20 min. To cause a color reaction in glucose, 0.1 mL of a reaction mixture was added to 3.0 ml of a quantitative glucose reagent (Glucose CII Test Wako, FUJIFILM Wako, Osaka, Japan) and incubated at 40°C for 30 min. Absorption was measured

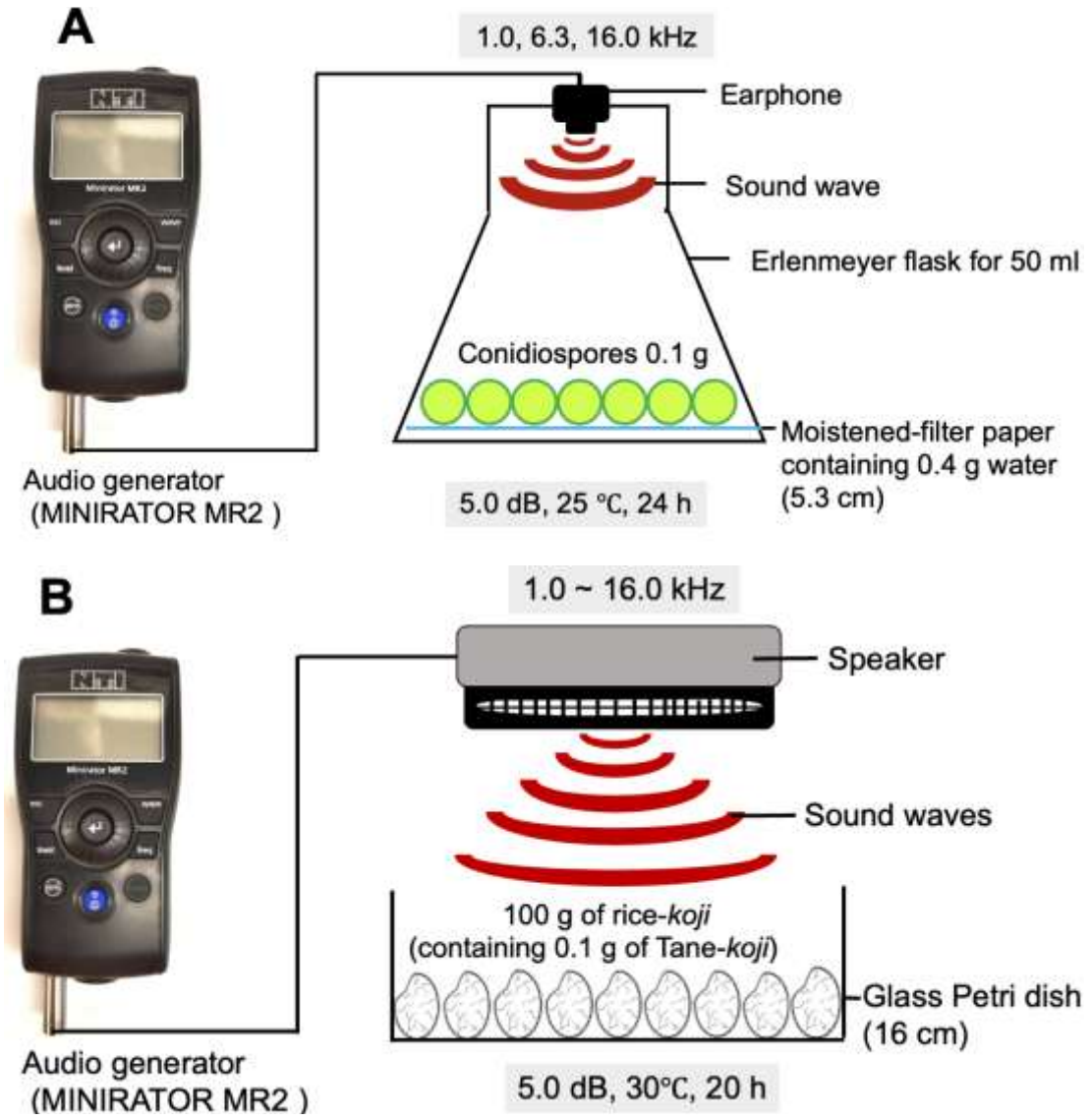

Figure 1. Methods of irradiating koji-*mold* with sound waves. “A” indicates the methods of irradiating *A. oryzae* RIB40 conidiospores with sound waves. To irradiate by sound waves, 0.1 g of conidiospores was aliquoted in a 50 ml Erlenmeyer flask with moistened filter paper on the bottom. The audio generator MINIRATOR MR2 was used to generate sound waves. The power level and frequency of sounds were adjusted. For the output of sounds, an earphone was connected to the head of the flask with vinyl tape. Conidiospores were irradiated with 1.0, 6.3, and 16 kHz sound waves at 25^{o}C for 24 h. “B” indicates the methods of irradiating conidiospores of Tane-*koji* that the stage of the hyphal elongation with sound waves. Rice-koji was prepared with 0.1 g of Tane-*koji* in Glass Petri dish of 16 cm in diameter at 30^{o}C for 20 h. After incubation for 20 h, sound waves was irradiated with the speaker that is connected to the audio generator MINIRATOR MR2 at 30^{o}C for 20 h. The power level and frequency of sounds were adjusted.
Source: Authors

at 505 nm with spectrophotometer U-1800 (Hitachi High-Tech Inc., Tokyo, Japan).

Determination of the acid protease activity of rice-*koji*

To reveal how the acid protease activity of rice-*koji* prepared with sound-irradiated conidiospores was changed, the acid protease activity of rice-*koji* was measured according to the official methods described in Taniguchi (1993). For the enzymatic reaction, 0.5 mL of properly diluted extract solution was mixed with 1.0 ml of 2.0% (w/v) casein solution (Nacalai Tesque, Kyoto, Japan) and incubated at 40°C for 60 min. To stop the enzymatic reaction, 3.0 mL of 0.4 M trichloroacetic acid solution (Nacalai Tesque, Kyoto, Japan) was added. The resulting mixture was filtered. To cause a color reaction in tyrosine, 1.0 mL of the filtrate was mixed with 5.0 ml of 2.2 M sodium carbonate solution and 1.0 ml of 0.9 N Folin-Ciocalteu Reagent (Nacalai Tesque, Kyoto, Japan). This mixture was

Table 1. Designed primers for each gene of *A. oryzae* RIB40

mRNA to be detected	Primer Name	Primer Sequence (5'-3')
AO090003000321	Aor_glaB_+488_FW	5'-TCGCATATGGCAACTCTCTG-3'
AO090003000321	Aor_glaB_+891_RV	5'-GCAGGGCTGGAATGTTGTAT-3'
AO090701000065	Aor_actA_+95_FW	5'-GTATCGTTCTGGATTCTGGTGAC-3'
AO090701000065	Aor_actA_+1333_RV	5'-AGAGATCCTTACGGACATCAACA-3'

Source: Authors

incubated at 40°C for 30 min in the dark. Absorption was measured at 660 nm with spectrophotometer U-1800 (Hitachi High-Tech Inc., Tokyo, Japan).

Extraction of total RNA from conidiospores

To reveal whether dormant stage of conidiospores respond to sound waves, we observed the accumulation of the expression product of *glaB* encoding glucoamylase. To extract total RNA, 0.1 g of sound-irradiated conidiospores was pulverized with a pestle and mortar cooled by liquid nitrogen. Total RNA was extracted according to instructions for the RNeasy Plant Mini Kit (QIAGEN, Tokyo, Japan). The quantity of prepared total RNA was determined by using a BioPhotometer (Eppendorf, Japan).

Semi-quantitative RT-PCR and electrophoresis

Primers for gene-encoding glucoamylase (*glaB*) were designed using Primer3Plus (https://www.primer3plus.com/). Genome sequences were referenced in the *Aspergillus* Data Base (http://www.aspgd.org/), and are currently available in the Fungi Data Base (https://fungidb.org/fungidb/app). Normalization of the expression level of the gene was performed with gene-coding actine (*actA)*. The expression was detected by PCR using a pair of oligonucleotide primers listed in Table 1. KOD-Plus-Neo (TOYOBO Co., Ltd., Osaka, Japan) was used as the DNA polymerase for PCR. Degradation of the DNA of the total RNA solution was performed in accordance with the instructions for Deoxyribonuclease (RT Grade) for Heat Stop (Nippon Gene, Tokyo, Japan). Reverse transcription reaction was performed in accordance with the instructions for PrimeScript IV First Strand cDNA Synthesis Mix (Takara Bio Inc., Kusatsu, Japan). Semi-quantitative RT-PCR was performed in accordance with the instructions for the Takara PrimeScript RT-PCR Kit (Takara Bio Inc., Kusatsu, Japan). To perform electrophoresis, agarose gel containing 1.0% (w/v) agarose was prepared with 0.5 × TAE buffer. Electrophoresis was performed using a WSE-1710 Submerge-Mini (ATTO, Tokyo, Japan) at a voltage of 50 V for 30 min. Gene Ladder Wide 1 (Nippon Gene, Tokyo, Japan) was used as a ladder marker. GX GelRed Prestain Loading Buffer with Blue (Cosmo Bio, Tokyo, Japan) was used to stain samples, and the fluorescence of samples was detected by Gel Scene GS-GU (ASTEC, Fukuoka, Japan). To quantify the expression levels of *glaB*, qTOWER^{3}G Real-Time Thermal Cycler (Analytik Jena, Kanagawa, Japan) was used to perform real-time PCR. PCR reaction was performed in accordance with the protocol of TB Green Premix Ex Taq^{TM} II (Takara, Tokyo, Japan).

Microscopic observation of conidiospores

To reveal how the germination ratio of conidiospores was changed by sound waves, the conidiospores were observed microscopically in accordance with the protocol for the Thoma hemocytometer (Minato Medical, Tokyo, Japan). To incubate rice-*koji,* 0.2 g of sound-irradiated conidiospores was inoculated onto 10 g of steamed rice. To collect conidiospores, 0.5 g of the resulting rice-*koji* was recovered in test tubes at 0, 2, 4, 6, and 8 h after incubation and suspended with 0.2 ml of deionized water containing 0.1% (v/v) Tween 80 (Nacalai Tesque, Kyoto, Japan) and stirred. The supernatant obtained by stirring was collected and diluted threefold with prepared deionized water in another test tube. To observe the conidiospores under a glass cover, 20 µl of the resulting suspension was placed on the center of the Thoma hemocytometer. An Olympus BH-2 Microscope (Olympus, Tokyo, Japan) was used for microscopy.

Statistical significance test

The difference between the sound and non-sound conditions was examined by a statistical significance test (*t*-test) (Sakoda et al., 1954). Each value is presented as the mean ± standard deviation of three independent experiments (n = 3). The error range was calculated from the standard deviation of three samples. A significant difference was recognized when the one-sided test was less than 0.05 ($P < 0.05$).

RESULTS

Effects of sound waves on the expression level of *glaB*

During the germination stage, conidiospores were irradiated at 1.0, 6.3, and 16 kHz for 24 h on the moistened filter paper. Then, we determined to observe the changes in the expression level of *glaB* and compare it with that of conidiospores not irradiated by sound.

When conidiospores were irradiated with sound waves at 1.0 kHz, the expression level of *glaB* was increased as compared with that of conidiospores that had not been irradiated by sound. The quantitative result of real-time PCR indicated that the expression level of *glaB* was not changed (data not shown).

When conidiospores were irradiated with sound waves at 6.3 kHz, the expression level of *glaB* was not changed as compared with that of conidiospores not irradiated by sound. The quantitative result of real-time PCR indicated that the expression level of *glaB* was decreased by 0.75 times as compared with that of conidiospores not irradiated by sound (data not shown).

When conidiospores were irradiated with sound waves at 16 kHz, the expression level of *glaB* was suppressed

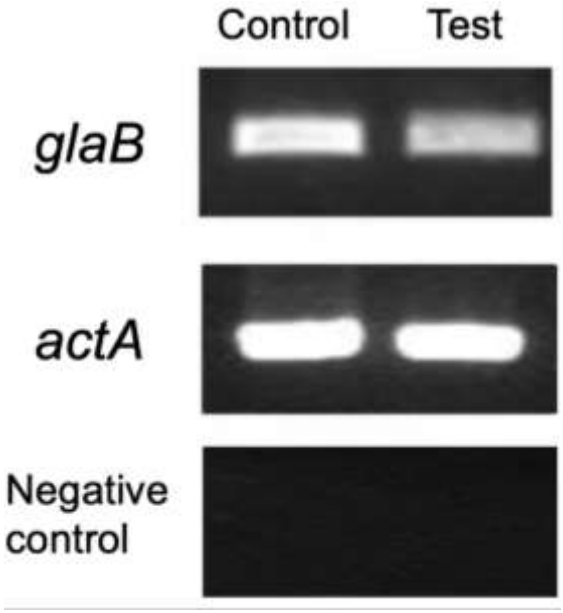

Figure 2. Effects of sound waves on the expression level of *glaB* encoding glucoamylase in *Aspergillus oryzae* RIB40. The gene expression levels were normalized by the expression level of *actA* and were evaluated via semi-quantitative RT-PCR analysis. The mRNA extracted from conidiospores not irradiated by sound is labeled “Control.” The mRNA extracted from conidiospores sound irradiated at 16 kHz is labeled “Test.” Total RNA of which reverse transcriptional reactions were performed without reverse transcriptase is labeled "Negative control" to ensure that contaminant DNA was not present in the samples.
Source: Authors

as compared with that of conidiospores not irradiated by sound (Figure 2). The quantitative real-time PCR result indicated that the expression level of *glaB* was changed by 1.28 times as compared with that of conidiospores not irradiated by sound (data not shown).

These results suggest that the expression level of *glaB* showed different changes in response to sound waves at different frequencies.

Effects of sound waves on glucoamylase activity

Rice-*koji* was prepared with conidiospores irradiated by sound waves during the germination stage for 24 h on moistened filter paper. Then we determined to observe the changes in the glucoamylase activity of rice-*koji* prepared with sound-irradiated conidiospores and compared with those of rice-*koji* prepared with conidiospores not irradiated by sound. Rice-*koji* was collected at 24 and 36 h after inoculation, and changes in the glucoamylase activity over time were observed during the rice-*koji*-making process (Figure 3).

In the case of sound waves at 1.0 kHz, the glucoamylase activities of rice-*koji* prepared with conidiospores not irradiated by sound (that is, in the control condition) were 164 ± 46.7 and 444 ± 83.4 (units/g of rice-*koji*), respectively. When conidiospores were irradiated with sound waves at 1.0 kHz, the glucoamylase activities were, respectively, 1.1, 1.1 times higher than those of rice-*koji* prepared with conidiospores not irradiated by sound. A significant difference was not observed, and glucoamylase activity was enhanced at 24 and 36 h after inoculation. It was suggested that irradiating sound waves at 1.0 kHz may enhance the glucoamylase activity of rice-*koji.*

In the case of sound waves at 6.3 kHz, the glucoamylase activities of rice-*koji* prepared with conidiospores not irradiated by sound were 112 ± 33.3 and 314 ± 23.1 (units/g of rice-*koji*), respectively. When conidiospores were irradiated with sound waves at 6.3 kHz, glucoamylase activities were, respectively, 0.9 and 1.2 times changed as compared with those of rice-*koji* made with conidiospores not irradiated by sound. An increase in the glucoamylase activity was observed during the first 36 h after incubation.

In the case of sound waves at 16 kHz, the

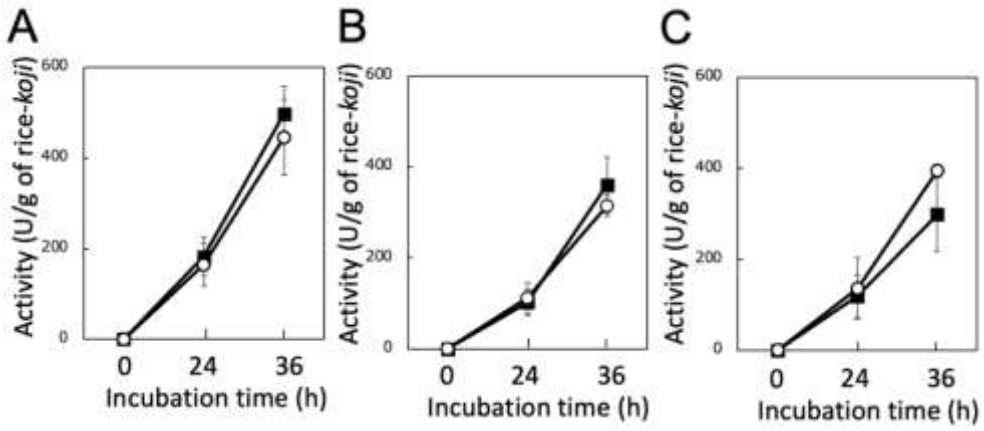

Figure 3. Changes in the glucoamylase activity of *A. oryzae* RIB40. Circles indicate the glucoamylase activity of rice-*koji* prepared with conidiospores not irradiated by sound (that is, the control condition). Black squares indicate the glucoamylase activity of rice-*koji* prepared with sound-irradiated conidiospores at frequencies of 1.0, 6.3, and 16 kHz (A, B, and C, respectively) for 24 h. Vertical axes indicate activity showing the color reaction of a 1 µg/ml glucose equivalent at 40℃ for 60 min from soluble starch, defined as (units/g of rice-*koji*). The horizonal axis indicates time after incubation to make rice-*koji* was started. Absorption was measured at 505 nm.
Source: Authors

glucoamylase activities of rice-*koji* prepared with conidiospores not irradiated by sound were 143 ± 62.3 and 391 ± 12.5 (units/g of rice-*koji*), respectively. When conidiospores were irradiated with sound waves at 16 kHz, glucoamylase activities were, respectively, 0.9 and 0.8 times less than that of rice-*koji* prepared with conidiospores not irradiated by sound. A significant difference was not observed, glucoamylase activity was decreased at 24 and 36 h after inoculation.

These results suggest that sound waves given to conidiospores during the germination stage affect the glucoamylase of rice-*koji.* Moreover, these results suggest that the effects of sound waves were reflected in the glucoamylase activity from 24 to 36 h after inoculation.

Effects of sound waves on acid protease activity

Before the rice-*koji*-making process, conidiospores during the germination stage were irradiated with sound waves for 24 h on moistened filter paper in advance. We determined to observe the changes of the acid protease activity of rice-koji prepared with sound-irradiated conidiospores as compared with those of rice-*koji* prepared with conidiospores that had not been irradiated by sound. Rice-koji was collected at 24 and 36 h after inoculation, and changes in the acid protease activity over time during the rice-*koji*-making process were observed (Figure 4).

In the case of sound waves at 1.0 kHz, the acid protease activities of rice-*koji* prepared with conidiospores not irradiated by sound (that is, the control condition) were 58.8 ± 9.90 and 90.7 ± 7.68 (K units/g of rice-*koji*), respectively. When conidiospores were irradiated with sound waves at 1.0 kHz in advance, the acid protease of rice-*koji* was not changed at any time.

In the case of sound waves at 6.3 kHz, the acid protease activities of rice-*koji* prepared with conidiospores not irradiated by sound were 49.2 ± 8.04 and 94.9 ± 3.89 (K units/g of rice-*koji*), respectively. When conidiospores were irradiated with sound waves at 6.3 kHz, the acid protease activity was 6.3 kHz in advance, and the acid protease of rice-*koji* was not changed at any time.

In the case of sound waves at 16 kHz, the acid protease activities of rice-*koji* prepared with conidiospores not irradiated by sound wave were 47.1 ± 4.83 and 83.7 ± 5.02 (K units/g of rice-*koji*), respectively. When conidiospores were irradiated with sound waves at 16 kHz in advance, the acid protease activity of rice-*koji* was not changed at any time.

Effects of sound waves on germination ratio

To reveal the effects of sound waves on conidiospores during the germination stage on the germination ratio of conidiospores, we determined to inoculate steamed rice with sound-irradiated conidiospores. We also compared that with the effects of conidiospores not irradiated by sound. The conidiospores were collected at 2, 4, 6, and 8 h after inoculation (Figure 5).

In the case of sound waves at 1.0 kHz, the germination ratios of conidiospores not irradiated by sound were 0.67 ± 0.12, 6.79 ± 7.64, 20.2 ± 5.22, 23.9 ± 10.7, and 26.6 ± 14.3%, respectively. When conidiospores were irradiated with sound waves at 1.0 kHz, the germination ratios were,

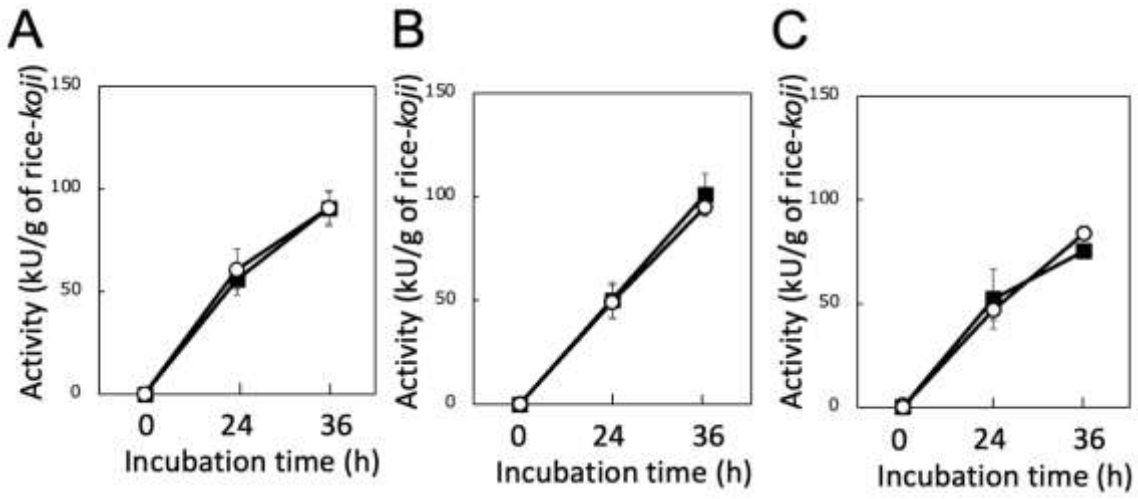

Figure 4. Changes in the acid protease activity of *A. oryzae* RIB40. Circles indicate the acid protease activity of rice-*koji* prepared with conidiospores not irradiated by sound (that is, the control condition). Black squares indicate the acid protease activity of rice-*koji* prepared with sound-irradiated conidiospores at frequencies of 1.0, 6.3, and 16 kHz (A, B, and C, respectively) for 24 h. Vertical axes indicate the activity showing the color reaction of a 1 µg/ml tyrosine equivalent at 40°C for 60 min from casein, defined as (K units/g rice-*koji*). The horizonal axes indicate time after incubation to make rice-*koji* was started. Absorption was measured at 660 nm.
Source: Authors

respectively, 0.9, 1.6, 0.7, 0.7, and 1.0 times changed as compared with those conidiospores not irradiated. It was suggested that sound waves at 1.0 kHz may suppress the germination ratio from 4 to 6 h after inoculation and the effect of sound waves on the germination ratio was lost by 8 h after incubation.

In the case of sound waves at 6.3 kHz, the germination ratios of conidiospores not irradiated by sound were 0.81 ± 0.12, 2.42 ± 0.51, 8.77 ± 3.82, 10.4 ± 2.26, and 13.0 ± 0.87%, respectively. When conidiospores were irradiated with sound waves at 6.3 kHz, the germination ratios were, respectively, 0.7, 1.1, 0.9, 0.8, and 0.9 times changed as compared with those of non-irradiated conidiospores. It was suggested that sound waves at 6.3 kHz have a slight inhibitory effect on the germination ratio from 4h to 6 h after inoculation.

In the case of sound waves at 16 kHz, the germination ratios of conidiospores not irradiated by sound were 0.71 ± 0.17, 3.90 ± 2.62, 15.8 ± 5.94, 15.3 ± 5.07, and 19.2 ± 7.62%, respectively. When conidiospores were irradiated with sound waves at 16 kHz, the germination ratios were, respectively, 0.9, 0.8, 1.2, 1.5, and 1.4 times changed as compared with those of conidiospores not irradiated by sound wave. It was suggested that sound waves at 16 kHz may enhance the germination ratio from 4 to 8 h after inoculation. A significant difference was not observed.

These results suggest that sound waves given to conidiospores during the germination stage affect the germination ratio. Moreover, it was suggested that the effect of sound waves were reflected in the germination ratio from 4 to 6 h after inoculation, and the effect of sound waves at 16 kHz was maintained even after 6 h after inoculation.

Effects of sound waves on hyphal weight

To reveal the effects of sound waves on the hyphal elongation of conidiospores during the later stage of growing on steamed rice, we determined to observe the changes in hyphal weight of rice-*koji* prepared with sound-irradiated conidiospores as compared with those of rice-*koji* that had not been irradiated by sound. Rice-*koji* was collected at 24 and 36 h after inoculation (Figure 6).

In the case of sound waves at 1.0 kHz, the hyphal weights of rice-*koji* prepared with conidiospores not irradiated by sound wave were 7.47 ± 1.61 and 15.2 ± 1.00 (µg/g of rice-*koji*), respectively. When conidiospores were irradiated with sound waves at 1.0 kHz in advance, the hyphal weight of rice-*koji* was not changed at any time.

In the case of sound waves at 6.3 kHz, the hyphal weights of rice-*koji* prepared with conidiospores not irradiated by sound wave were 5.59 ± 0.37 and 9.27 ± 0.53 (µg/g of rice-*koji*), respectively. When conidiospores were irradiated with sound waves at 6.3 kHz in advance, the hyphal weight of rice-*koji* was not changed at any time.

In the case of sound waves at 16 kHz, the hyphal weights of rice-*koji* prepared with conidiospores not irradiated by sound wave 6.70 ± 1.54 and 11.4 ± 0.21 (µg/g of rice-*koji*), respectively. When conidiospores were

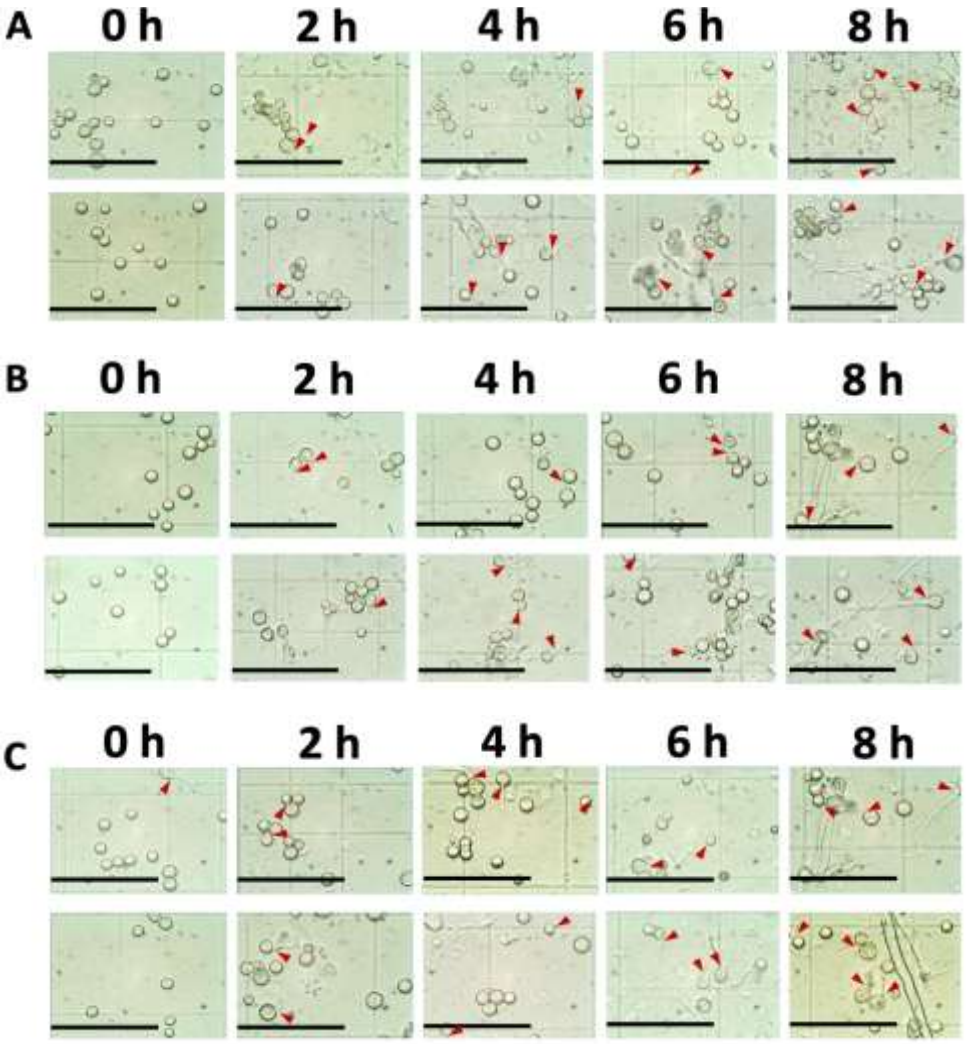

Figure 5. Changes in the germination ratio of conidiospores of *A. oryzae* RIB40. The upper panels show conidiospores not irradiated by sound (that is, the control condition) at 0, 2, 4, 6, and 8 h after the incubation to make rice-*koji* was started. The lower panels show sound-irradiated conidiospores at frequencies of 1.0, 6.3, and 16 kHz (A, B, and C, respectively) for 24 h. The black bars indicate 50 µm. The triangles indicate germinated cells.
Source: Authors

irradiated with sound waves at 16 kHz in advance, the hyphal weights were, respectively, 1.1 and 1.2 times higher than those of rice-*koji* prepared with conidiospores not irradiated by sound. A significant difference was observed ($P < 0.05$) in the hyphal weight at 36 h after incubation.

These results suggest that sound waves at 16 kHz given to conidiospores during the germination stage affect the hyphal elongation from 24 to 36 h after inoculation.

DISCUSSION

The effects of sound waves on hyphal weight, enzyme activities, and germination ratio at each frequencies are summarized in Tables 2 to 5. When the effects of sound waves on each parameter were compared, interesting correlations were found.

Correlation between the expression level of *glaB* and glucoamylase activity

It was reported that the expression level of *glaB* that codes glucoamylase was not usually changed from the dormant stage to the germination stage of conidiospores and was enhanced at the hyphal elongation stage, which is a later phase of conidiospores in *Aspergillus* spp. (Leeuwen et al., 2012). In the hyphal elongation

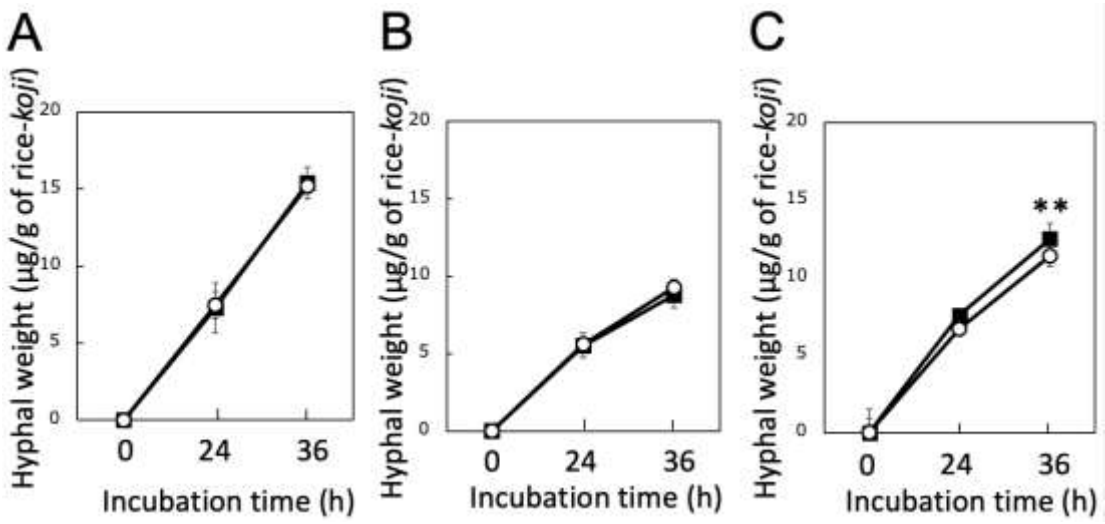

Figure 6. Changes in the hyphal weight of *A. oryzae* RIB40. Circles indicate the hyphal weight of rice-*koji* prepared with conidiospores that were not irradiated by sound (that is, the control condition). Black squares indicate the hyphal weight of rice-*koji* prepared with sound-irradiated conidiospores at frequencies of 1.0, 6.3, and 16 kHz (A, B, and C, respectively) for 24 h. Vertical axes indicate the hyphal weight contained in 1.0 g of rice-*koji*, defined as (µg/g of rice-*koji*). Asterisks (**) indicate a significant difference ($P < 0.05$). The horizonal axes indicate time after incubation to make rice-*koji* was started. Absorption was measured at 585 nm.
Source: Authors

Table 2. The ratio of changes in glucoamylase activity.

Frequency (kHz)		Period (h)
		24-36
1.0	Control	2.71 ± 0.40
	Test	2.73 ± 0.38
6.3	Control	2.80 ± 1.01
	Test	3.53 ± 1.41
16	Control	2.71 ± 1.17
	Test	2.27 ± 0.89

"Control" indicates the glucoamylase activity of rice-*koji* prepared with conidiospores not irradiated by sound. "Test" indicates the glucoamylase activity of rice-*koji* prepared with sound-irradiated conidiospores. Numbers indicate the ratios of glucoamylase activity increases produced from 24 to 36 h after inoculation to make rice-*koji*.
Source: Authors

stage of *koji*-mold, it was reported that the expression level of *glaB* was regulated by transcriptional factors, such as AmyR and FlbC (Gomi et al., 2000). According to Gomi et al. (2000), in eutrophic conditions, when transcriptional factor AmyR was activated by isomaltose, the expression levels of genes that codes starch hydrolytic enzymes, such as *amyA/B/C, glaA/B,* and *agdA,* were enhanced by activated AmyR. In this study, we suggested that during the germination stage, conidiospores were irradiated by sound waves at 16 kHz for 24 h on moistened filter paper; the expression level of *glaB* was suppressed as compared with that of conidiospores that had not been irradiated by sound. This result suggests that the expression level of *glaB* was unchanged from the dormant stage to the germination stage of conidiospores but was changed by irradiating sound waves. Quantitative results of real-time PCR indicated a 1.28-times changes in the expression level of *glaB* as compared with that of conidiospores that had not been irradiated by sound. Because the scale of changes was considered to be small, we would like to continue the experiment to confirm the decrease in the expression level of *glaB* by real-time PCR as well.

When the changes of glucoamylase activities of rice-

Table 3. The ratio of changes in acid protease activity.

Frequency (kHz)		Period (h)
		24-36
1.0	Control	1.49 ± 0.28
	Test	1.62 ± 0.01
6.3	Control	1.93 ± 0.54
	Test	2.01 ± 0.67
16	Control	1.78 ± 0.25
	Test	1.45 ± 0.37

"Control" indicates the acid proteases of rice-*koji* prepared with conidiospores not irradiated by sound. "Test" indicates the acid proteases of rice-*koji* prepared with sound-irradiated conidiospores. Numbers indicate the ratios of acid protease activity increases produced from 24 h to 36 h after inoculation to make rice-*koji*.
Source: Authors

Table 4. The ratio of changes in conidial germination.

Frequency (kHz)		Period (h)			
		0-2	2-4	4-6	6-8
1.0	Control	10.1 ± 1.08	2.97 ± 0.61	1.18 ± 0.24	1.11 ± 0.09
	Test	12.1 ± 6.50	2.03 ± 1.15	1.24 ± 0.25	1.44 ± 0.24
6.3	Control	2.97 ± 0.67	3.63 ± 2.27	1.19 ± 0.60	1.25 ± 0.20
	Test	4.78 ± 3.15	2.88 ± 0.93	1.05 ± 0.49	1.57 ± 0.23
16	Control	5.52 ± 5.75	4.06 ± 1.06	1.00 ± 0.08	1.25 ± 0.09
	Test	4.69 ± 0.67	5.99 ± 1.49	1.21 ± 0.09	1.20 ± 0.13

"Control" indicates the germination ratio of conidiospores not irradiated by sound. "Test" indicates the germination ratio of sound-irradiated conidiospores. Numbers indicate the ratio of increase in conidial germination from the start of the rice-*koji*-making process to 0, 2, 4, 6 and 8 h afterward.
Source: Authors

Table 5. The ratio of changes in hyphal weight.

Frequency (kHz)		Period (h)
		24-36
1.0	Control	2.03 ± 0.13
	Test	2.12 ± 0.25
6.3	Control	1.66 ± 0.07
	Test	1.59 ± 0.07
16	Control	1.65 ± 0.37
	Test	1.70 ± 0.20

"Control" indicates the hyphal weight of rice-*koji* prepared with conidiospores not irradiated by sound. "Test" indicates the hyphal weight of rice-*koji* prepared with sound-irradiated conidiospores. Numbers the ratio of changes in hyphal weight of rice-*koji* from 24 h to 36 h after inoculation to make rice-*koji*.
Source: Authors

koji prepared with conidiospores not irradiated by sound were observed, it was found that the largest increase of glucoamylase activity was observed from 24 to 36 h after inoculation (Table 2). In the case of sound waves at 16 kHz, the ratio of increase in glucoamylase activity from 24 to 36 h after inoculation was suppressed as compared with that of rice-*koji* prepared with conidiospores that had not been irradiated by sound.

In the case of rice-*koji* prepared with conidiospores not irradiated by sound, the expression level of *glaB* was relatively enhanced accompanied by the increase of glucoamylase activity of rice-*koji* from 24 to 36 h after inoculation. However, due to the irradiating sound waves at 16 kHz on conidiospores during the germination stage, the rice-*koji*-making process begin with a suppressed expression level of *glaB*. Therefore, it was considered that when sound-irradiated conidiospores were incubated in eutrophic conditions, such as those found in steamed rice, the glucoamylase activity of rice-*koji* was also relatively decreased as compared with that of rice-*koji* not irradiated by sound. These results suggest that sound waves given to conidiospores during the germination stage affect the glucoamylase activity of rice-*koji* prepared with sound-irradiated conidiospores.

Effects of sound waves on glucoamylase activity in relation to hyphal weight

From the result that the glucoamylase activity of rice-*koji* prepared with sound-irradiated conidiospores was changed as compared with that of rice-*koji* prepared with conidiospores that had not been irradiated by sound, the hyphal weight was also relatively changed. Therefore, we determined to reveal the correlation between hyphal weight and the glucoamylase activity of rice-*koji.*

It was reported that hyphal weight usually began to increase 20 h after inoculation and continued to increase exponentially until 40 h after inoculation (Yoshii et al., 1973). After 40 h, the hyphal weight transitioned to the stationary phase, and the changes in hyphal weight slowed. In this study, we observed that the hyphal weight of rice-*koji* prepared with conidiospores not irradiated by sound increased from 24 to 36 h after inoculation (Table 5). When conidiospores were irradiated by sound waves in advance, the hyphal weight of rice-*koji* prepared with sound-irradiated conidiospores was not changed as compared with that of rice-*koji* prepared with conidiospores that had not been irradiated by sound. From this result, we found that sound waves given to conidiospores during the germination stage did not affect the hyphal cell growth of rice-*koji*. In the case of the effect of sound waves at 16 kHz, as compared with changes in the glucoamylase activity, as shown in Figure 3, glucoamylase activity was suppressed from 24 h after inoculation as compared with that of rice-*koji* prepared with conidiospores that had not been irradiated by sound, even though hyphal cell growth had not changed. The correlation between hyphal cell growth and the glucoamylase activity of *koji*-mold has been confirmed (Iwano et al., 2002). In this study, we observed that the glucoamylase activity in relation to the hyphal weight of rice-*koji* prepared with conidiospores not irradiated by sound increased over time. When rice-*koji* was prepared with conidiospores sound irradiated at 16 kHz, it was found that the glucoamylase activity in relation to the hyphal weight of rice-*koji* was suppressed over time during the rice-*koji*-making process. From these results, by irradiating conidiospores with sound waves, the ability to produce glucoamylase in relation to the hyphal cell growth of rice-*koji* was suppressed.

Moreover, according to the results shown in Figure 6, even though hyphal cell growth was not changed, the hyphal weight of rice-*koji* prepared with conidiospores sound irradiated at 16 kHz was increased from 24 h after inoculation as compared with that of rice-*koji* prepared with conidiospores that had not been irradiated by sound. This result suggests that, by irradiating conidiospores with sound waves at 16 kHz, the increase in the hyphal weight of rice-*koji* prepared with sound-irradiated conidiospores may begin at a point in time before 20 h.

Effects of sound waves on acid protease activity in relation to hyphal weight

The effects of sound waves on acid protease activity in relation to the hyphal weight of rice-*koji* prepared with sound-irradiated conidiospores were investigated. It was reported that acid protease activity was usually increased from 16 h after inoculation and was actively enhanced from 20 to 32 h after inoculation, and then transitioned to the stationary phase, when the changes of acid protease activity became slower (Yoshii et al., 1973). In this study, we observed that the acid protease activity of rice-*koji* prepared with conidiospores not irradiated by sound was increased from 24 to 36 h (Table 3).

As compared with acid protease activity of rice-*koji* prepared with sound-irradiated conidiospores, it was found that the ratio of increase of acid protease activity was changed from 24 to 36 h after inoculation. The effects of sound waves were different according to its frequency. In the case of 1.0 kHz, the ratio of the increase in acid protease activity was enhanced as compared with that of rice-*koji* prepared with sound-irradiated conidiospores from 24 to 36 h after inoculation. In the case of 16 kHz, the ratio of increase of acid protease activity was suppressed as compared with that of rice-*koji* prepared with sound-irradiated conidiospores from 24 to 36 h after inoculation. It was considered that the changes in the acid protease activity of rice-*koji* become slower 32 h after inoculation with or without irradiating sound waves.

As compared with the hyphal weight results, even though the hyphal elongation stage remained constant with and without sonication (Table 5), the ratio of the

increase in acid protease activity of rice-*koji* prepared with sound-irradiated conidiospores at 16 kHz was suppressed from 24 to 36 h after inoculation. This result indicated that the acid protease activity in relation to hyphal weight was changed by irradiating sound waves on the conidiospores during the germination stage.

Correlation between germination ratio and hyphal weight

From the results of Figure 6 and Table 5, when conidiospores were irradiated by sound waves at 16 kHz, the hyphal elongation of rice-*koji* prepared with sound-irradiated conidiospores took place earlier than that of rice-*koji* prepared with conidiospores that had not been irradiated by sound. We investigated the correlation between the germination ratio and hyphal weight.

When conidiospores of *Aspergillus* spp. were exposed to a eutrophic environment with adequate water and temperature, such as complete medium (CM), during the dormant stage for 2 h after inoculation, the conidiospores were reported to have moved into the isotropic growth stage after 4 h. The germination stage began after 6 h, and the polarized growth stage took place at the end of an 8 h period (Leeuwen et al., 2012). When conidiospores were irradiated by sound waves on moistened filter paper, the germination ratio was between 0.5 and 2.5%. These results showed that the sound-irradiated conidiospores on the moistened filter paper correspond to the isotropic growth stage while preparing for germination to the early germination stage. When steamed rice was inoculated with non-irradiated conidiospores placed on moistened filter paper for 24 h, the conidial germination stage was observed 2 h after inoculation, and the hyphal elongation stage was observed 6 h after inoculation (Figure 5). It was also found that the largest increase in the germination ratio was observed from 0 to 4 h after inoculation (Table 4). When conidiospores were irradiated by sound waves at 16 kHz during the germination stage on moistened filter paper for 24 h, it was found that the germination ratio increase was enhanced as compared with that of conidiospores not irradiated by sound from 4 h after inoculation on steamed rice. On the other hand, according to the result earlier stated (effects of sound waves on the germination ratio), the germination ratio of sound-irradiated conidiospores was enhanced as compared with that of non-irradiated conidiospores 4 h after inoculation on steamed rice. From these results, we suggested that by irradiating conidiospores with 16 kHz sound waves during the germination stage, their germination ratio was enhanced, and hyphal elongation occurred at an earlier stage than that of non-irradiated conidiospores. Therefore, we considered that the amount of hyphal weight during the same rice-*koji*-making may be enhanced.

It was also suggested that the expression level of genes related to germination, e.g., *flbC* and *medA* may be affected by sound waves. It was reported that *flbC* works as a positive regulator of *brlA,* and *med*A is a gene involved in the beginning of germination that was regulated by transcriptional activator BrlA (Leeuwen et al., 2012). The expression level of *medA* was increased during the germination process from 2 to 6 h after inoculation. When conidiospores were irradiated by 16 kHz sound waves, the germination ratio was increased from 2 h after incubation. Therefore, it was indicated that the expression level of *medA* also may be increased by sound-wave irradiation. An experiment designed to investigate the effects of sound waves on the expression levels of genes involved in the beginning of germination is being planned.

Conclusion

These results suggest that sound waves may be an important environmental factor in the rice-*koji*-making process. When conidiospores were irradiated by sound waves during the germination stage, the expression level of *glaB* was changed as compared with that of conidiospores not irradiated by sound. This result suggests that conidiospores may respond to sound waves during the germination stage.

Moreover, it was also suggested that the hyphal weight and enzyme activity of rice-*koji* prepared with sound-irradiated conidiospores were changed as compared with those of rice-*koji* prepared with conidiospores not irradiated by sound. This suggests that sound waves during the germination stage of conidiospores affected the hyphal elongation and enzyme activity of rice-*koji* when sound-irradiated conidiospores were added on steamed rice. In the case of sound waves at 16 kHz, the germination ratio of conidiospores was enhanced and hyphal elongation occurred at an earlier stage than that of conidiospores not irradiated by sound; and the ability to produce glucoamylase in relation to hyphal cells of rice-*koji* was suppressed.

These results may be applied to the preparation of rice-*koji* that exhibits the desired enzyme activity. We think these results can contribute to the development of a new technology of rice-*koji*-making that controls the hyphal elongation and enzyme activity of koji-*mold* via irradiating of sound waves. Further work is needed to elucidate the detailed mechanisms of the response to specific frequencies of sound waves. This research is expected to contribute to the practical application of adjunct technology that will improve the quality of brewed beverages.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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An optimized protocol for *in vitro* regeneration of tropical maize inbred lines through cell suspension and semi-protoplast cultures

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Maize being an important staple food crop is widely consumed in Kenya but its production remains low due to biotic and abiotic challenges that have not been addressed sufficiently through conventional breeding. This study sought to optimize the establishment of cell suspension and protoplast cultures for 10 tropical inbred maize genotypes in order to identify genotypes that can readily establish in liquid cultures and those whose cell suspensions and protoplasts are capable of *in vitro* regeneration. Callus were induced using immature zygotic embryos on Murashige and Skoog (MS) medium supplemented with 3 mg/L 2, 4-D, 5 mg/L dicamba or 5 mg/L picloram. Dicamba and picloram induced friable calli that readily dispersed into cell suspensions in liquid MS medium supplemented with 0.1 g/L asparagine and either 0.4 or 0.8 g/L proline. Cell growth was determined by packed cell volume (PCV) every seven days. The highest PCV (240 µl/ml) was recorded in genotype EO4 followed by CML 216 (188 µ/ml). Optimal growth was observed in cells maintained in MS Amended with 0.4 or 0.8 g/L of proline in combination with 0.1 g/L asparagine compared to medium without proline. It was also observed that cells cultured in media with reduced Ammonium Nitrate (12 fold reduction) recorded higher PCV values than controls. Protoplasts were generated from the resulting cells using 2% cellulase and 0.5% pectolyase in an enzyme digestion cocktail containing Mannitol and Calcium Chloride (MaCa) and washed in MS with vitamins containing Mannitol (MSMa). Only cell clusters of genotype EO4 gave rise to plants with a regeneration frequency of 42.51%. In conclusion, success was achieved in callus initiation, formation of cell suspension cultures and their eventual regeneration into whole plants for selected tropical maize genotypes.

Key words: Cell suspensions, callus, somatic embryos, packed cell volume, protoplasts.

INTRODUCTION

Maize (*Zea mays* L) is an important staple food crop that is widely consumed in sub-Saharan Africa. It is also the third most cultivated cereal crop in the world after rice and wheat (Ram, 2011) and a principal crop that is grown in many parts of the world as human food and animal feed (Yadava et al., 2017). The average maize yield per hectare in Kenya is quite low relative to the demand owing to existing constraints to production including

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abiotic challenges such as drought and biotic stresses such as disease, pests, and weeds (Anami et al., 2008; Kariuki, 2015) in addition to poor agronomic practices that result in loss of soil fertility (Thomson, 2008). Management of these constraints has been unsuccessful, mainly because of the use of conventional breeding to develop resistant varieties. Despite a certain degree of success, some of the generated varieties result in undesired traits due to linkage drag and lack of appropriate well defined parental germ lines (Anami et al., 2009). There is need, therefore, to complement such breeding-based strategies with efficient and reliable genetic engineering manipulations for maize. This technology has previously been used in development of germplasm resistant to various pests, diseases such as maize streak virus (MSV) and fungi producing aflatoxin (Masanga et al., 2015).

Efficient use of genetic engineering techniques in the improvement of maize is hampered by the *in vitro* recalcitrance frequently seen in most cereals making it difficult to induce embryogenic callus and eventual regeneration of maize especially in elite varieties (Ge et al., 2016). Despite this challenge, transformation and regeneration of tropical maize lines and other genotypes including open pollinated varieties and hybrids (Ombori et al., 2008, 2013) has been achieved, albeit with some difficulty (Muoma et al., 2008; Oduor et al., 2006; Ombori et al., 2008; Omer et al., 2008). Successful transformation utilizes immature zygotic embryos as explants for indirect transgene delivery often mediated by *Agrobacterium tumefaciens* (Akoyi et al., 2013; Anami et al., 2010; Mgutu et al., 2011; Ombori et al., 2008). For direct transgene delivery however, only *in vitro* established tissues are suitable, but these still must be derived from immature zygotic embryos (Anami et al., 2010; Bedada et al., 2014; Seth et al., 2012; Shepherd et al., 2009). Despite prolonged efforts by many researchers, development of genetic transformation techniques for major cereal crops has not been fast enough. The recalcitrance of maize and most other monocot species to *Agrobacterium*-mediated transformation has necessitated advances in using direct DNA delivery methods (Bommineni and Jauhar, 1997; Gelvin, 1998). Unfortunately, preparatory procedures and the resultant transgenic lines possess redundant background genes that globally evoke biosafety and health concerns (Wang et al., 2000). The maize lines developed, therefore, require rigorous biosafety checks further delaying the rewards otherwise presented by biotechnology. Optimization and establishment of tissue culture protocols for tropical maize is imperative for genetic transformation in maize (Anami et al., 2010). In this paper, establishment of cell suspensions that provide a uniform donor explant material for *in vitro* manipulation of tropical maize genotypes was reported. Plant cell suspension cultures are dedifferentiated; hence, undergo much cell division and DNA synthesis availing the necessary cellular protein repertoire and conditions for easy integration of foreign naked DNA into the host genome. Most direct DNA transfer methods work efficiently when single cells are used as compared to use of differentiated explants (Schuurink and Louwerse, 2000). The cell population in suspension cultures is homogenous making it easy for cells to rapidly access nutrients, plant growth regulators (PGRs), and signals (Mustafa et al., 2011). The plant cells are totipotent, every cell contains all genetic information needed to grow into a new plant, hence any part of the plant can be used as an explant (Endress, 1967). Most maize tissue culture studies that have been done using cell suspensions and protoplast cultures for regeneration and transformation, are mainly for temperate genotypes (Boulton et al., 1993; Hu et al., 2020; Ortiz-Ramírez et al., 2018; Wang et al., 2000).

Despite the enormous progress achieved in this area, several challenges still remain, the main one being the recalcitrance of some protoplast systems, thus inability to express their totipotency. While a lot of investigations have been carried out on tropical maize to develop efficient tissue culture techniques using immature zygotic embryos as the preferred ex plants (Anami et al., 2010; Bedada et al., 2014; Binott et al., 2008; Oduor et al., 2006; Ombori et al., 2008); the pace has been and still is agonizingly slow (Mgutu et al., 2011) and little has been carried out to optimize the culture conditions for cell suspensions and protoplasts for regeneration and transformation. One of the reasons for this predicament is the lack of high throughput optimization of tissue culture protocols for tropical white maize, techniques that should be locally developed or accustomed and therefore suitable for tropical adopted maize genotypes. This is in contrast to temperate maize genotypes, which have remained reference benchmarks for tropical maize tissue culture procedures (Anami et al., 2010). Despite a lot of research that has been done on the genetic transformation of temperate maize genotypes, there is limited information available on genetic manipulation of tropical maize genotypes using cell suspensions and direct gene delivery (Kausch et al., 2021; Yadava et al., 2017).

Protoplasts from the maize endosperm cells have been used for introduction of DNA, RNA and Ribonuclear proteins (RNPs) using protoplast technologies in genome editing (Hu et al., 2020; Kausch et al., 2019; Songstad et al., 2017). To the best of our knowledge, this is the first attempt to establish cell suspension cultures using the selected tropical maize inbred lines and therefore sets up a platform for use in genetic transformation which in turn will open up the use of more cell based technologies for increased productivity of maize in the tropics. The protocol developed from this study will make possible cell manipulation with an aim of increasing yields and management of various biotic factors that affect maize production in different parts of the world but especially

Table 1. Formulations of media regimes for determination of packed cell volume in maize.

Auxin	Treatment	MS basal salts (g/L)	Proline (g/L)	Asparagine (g/L)
	D1	4.3	1.6	0.1
2,4-D	D2	4.3	0.8	0.1
	D3	4.3	0	0.1
	P1	4.3	1.6	0.1
Picloram	P2	4.3	0.8	0.1
	P3	4.3	0	0.1

D1: MS + 2 mg/L 2,4-D, 0.8 g/L proline + 0.1 g/L asparagine D2: MS + 2 mg/L 2,4-D, 0.4 g/L proline + 0.1 g/L asparagine D3: MS+ 2 mg/L 2,4-D+0.1 g/L asparagine P1: MS + 3 mg/L picloram, 0.8 g/L proline + 0.1 g/L asparagine P2: MS + 3 mg/L picloram, 0.4 g/L proline + 0.1 g/L asparagine P3: MS + 3mg/L picloram+0.1 g/L asparagine.

the tropics and Kenya in particular.

MATERIALS AND METHODS

Plant and explant generation

To generate immature zygotic embryos for use as explants, 10 tropical maize inbred lines, CML 216, CML 144, CML 395, acquired from the International Maize and Wheat Improvement Center (CIMMYT), and EO4, TL 21, TL 22, TL 26, TL 27, AO4 and TO4 obtained from Kenya Agricultural and Livestock Research Organization (KALRO), were planted at Kenyatta University research farm. At the flowering stage, tassels were covered with pollination bags (10 cm × 15 cm × 30 cm) while emerging silks were covered using clear polythene bags (15 cm × 15 cm) to prevent cross pollination. Controlled self-pollination was then done to obtain immature zygotic embryos.

Establishment of callus cultures

Maize cobs were harvested between 12 and 16 days after pollination and immature zygotic embryos aseptically extracted for use to initiate callus cultures *in vitro* using a protocol described by Negrotto et al. (2000). This was done on a callus induction medium (CIM) comprising 4.3 g/L Murashige and Skoog (MS) basal salts (Murashige and Skoog, 1962), 3% (w/v) sucrose, 0.8 mg/L silver nitrate, 100 mg/L casein hydrolysate and 2 mg/L 2, 4-dichlorophenoxyacetic acid (2,4-D). The pH of the media was adjusted to 5.8 using dilute HCL and NaOH followed by addition of 8% (w/v) plant agar prior to autoclaving at 121°C for 15 min. The cultures were maintained at 26 ± 2°C in the dark for two weeks then sub-cultured onto callus maintenance medium (CMM), similar to CIM but without silver nitrate. To aid in induction and maintenance of friable embryogenic callus, 3 mg/L dicamba and 1.0 mg/L kinetin were added to the media (Akoyi et al., 2013).

Establishment of cell suspensions

To establish cell suspension cultures, friable embryogenic calli initiated earlier were transferred into liquid medium comprising 4.3 g/L MS basal salts, 0.02 g/L thiamine, 1 g/L casein hydrolysate, 0.1 g/L myo-inositol, 3 mg/L picloram or 2 mg/L 2, 4 D, 1.16 g/L proline, 2 mg/L 2, 4-D, and 3% (w/v) sucrose (Wei and Xu, 1990). The pH of the media was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. 10 mL of the medium was dispensed into autoclaved 125 mL Erlenmeyer flasks and sealed with autoclaved cotton bulbs under aseptic conditions. Cell suspensions were initiated by placing 1, 2, 3, or 4 g of calli in the liquid medium, the flasks were sealed and incubated at 28°C on a rotary shaker at 100 to 120 revolutions per minute (rpm). Some of the cells developing in suspension were re-cultured onto solid CMM to form calli and somatic embryos that were subsequently used to regenerate clonal plantlets (Anami et al., 2010). Sub-culturing was done by refreshing the media after every 7 days and growth rate and general appearance of the cells monitored over time.

Determination of cell growth

To investigate cell growth in culture, packed cell volume (PCV) of the cells was determined by measuring the cell volume per 1 ml of cell suspension culture after allowing the cells to settle upon removing them from the rotary shaker followed by centrifugation at 100 to 120 rpm (David et al., 1989; Mustafa et al., 2011). Growth was first set at a PCV of 10 ± 2% of the total volume using fresh medium and then incubated at 28°C on a rotary shaker at 100 to 120 rpm. After every seven days, 1 mL of the cell suspension was aliquoted into microcentrifuge tubes and centrifuged at 6000 rpm, at room temperature, for 5 min and the supernatant discarded. The PCV was estimated using a graduated microcentrifuge tube and the data used to decipher the trend in growth rate of the cells in suspension. The PCV was determined using three replicates for each inbred line and media formulation. Several medium regimes (Table 1) were investigated to identify which formulations in the medium support the best growth of cells in suspension.

The medium was refreshed after every 7 days and growth rate of the cells monitored over time. For visual determination of growth, cells were observed under a bright-field microscope (Leica-Wetzlar, Germany) and photographed using a canon digital camera (Oita, Japan). To investigate the effect of ammonia on cell growth, different levels of ammonium nitrate were investigated. The amount of ammonium nitrate was reduced to 4, 8 and 12 fold (during media reconstitution) before initiation of cell suspensions. The PCV was then measured as earlier described and the results compared with those obtained using media with the normal ammonium nitrate (NH_4NO_3) concentration of 1650 mg/L (Jones, 2009).

Establishment of semi-protoplast cultures

Four-day old cells were used to generate semi-protoplasts through partial degradation of the cell wall using different combinations of cellulase and pectolyase enzymes. Cellulase was varied in a range of 1 to 3%, while pectolyase was maintained at 0.5% w/v

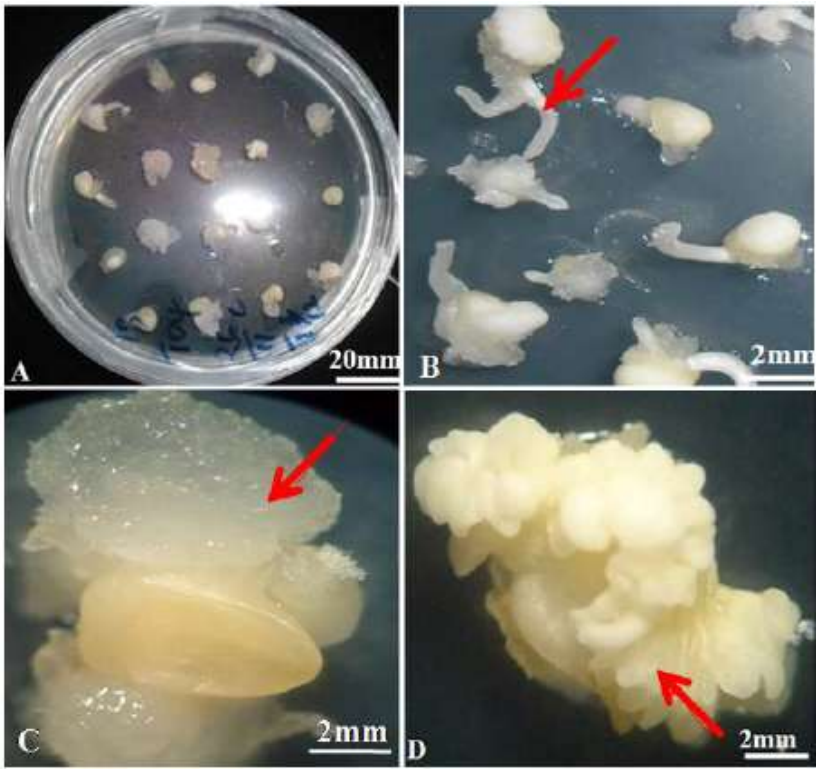

Figure 1. Establishment of friable embryogenic calli in tropical maize. (A) Immature zygotic embryos starting to form callus tissue on CIM. (B) Established callus showing precocious germination 14 days after callus initiation. (C) Nascent callus formation on CIM as seen under a light microscope (X 40 magnification). (D) Appearance of friable embryogenic callus on CMM with dicamba and kinetin.

concentration in the enzyme cocktail. The enzymes were first dissolved in 60 mM mannitol and 80 mM calcium chloride (MaCa) solutions at pH 4 (Armstrong et al., 1990; Boulton et al., 1993)

Ten milliliters of the enzyme solution was added to the cells in 50 mL sterile centrifuge tubes and the mixture incubated for 4 h at 28°C in the dark at 50 rpm. Cells were then harvested by centrifugation at 6000 rpm for 5 min (26 ± 2°C) and washed with fresh MS medium supplemented with 60 mM mannitol (MSMa). To ensure an equal amount of protoplasts was used, PCV of the cultures was set at 10% using fresh MSMa and the cells subsequently plated on semi-solid CMM containing 60 mM mannitol. Excess media was allowed to dry and the cultures were incubated in the dark at 26 ± 2°C to allow cell wall development. Samples of the cells were observed under a bright-field microscope (Leica Wetzlar, Germany) and photographs taken.

Whole plant regeneration from cell cultures

Cultures in suspension were first centrifuged at 6000 rpm for 10 min. The supernatant was then discarded and the collected cells and cell clusters plated on protoplast regeneration media (PRM) overlaid with autoclaved lens paper (Whatman No. 4, Sigma USA). The PRM comprised 4.4 g/L of MS salts with a fourfold ammonium nitrate reduction plus vitamins, 30 g/L sucrose, 2 mg/L 2, 4 D, 0.7 g/L proline, and 1.0 g/L casein hydrolysate. Cultures were incubated at 26±2°C in the dark for 3 to 4 weeks to allow for callus induction. Calli were transferred to callus maturation media (CMM), comprising 4.4 g/L MS with vitamins, supplemented with 60 g/L sucrose, 0.7 g/L proline and 1 mg/L NAA for 14 days. Callus showing the presence of somatic embryos were transferred onto regeneration medium (half strength MS salts and vitamins (2.2 g/L), 20 g/L sucrose and 8 g/L plant agar) for a further 2 weeks to allow for shoot formation and rooting. The number of shoots formed per callus were counted and recorded. *In vitro* regenerated plantlets with well-developed roots were transferred into 250 g pots containing autoclaved peat moss (Sungro horticulture, Amiran, Kenya), covered with wet/moisturized clear polyethylene bags and acclimatized for 10 days. Plants were then transferred to 10 kg potted soil and allowed to grow to maturity till seed formation in the green house at Kenyatta University.

One-way analysis of variance (ANOVA) of the frequencies (%) of callus induction, somatic embryogenesis and regeneration was performed using SAS version 9.2 and Tukey's HSD test at 95% confidence interval was used to determine significant differences among the means.

RESULTS

Initiation of friable embryogenic callus

Appearance of callus was varied for each genotype, notably callus appeared from the 5 to 7th day (Figure 1A). There was appearance of root-like structures on

Table 2. Frequencies of primary callus formation on CIM and friable embryogenic callus on CMM in maize inbred lines.

Maize ID	No. of embryos cultured	Callus induction frequency	Friable embryogenic callus frequency
CML 144	920	75.62±3.39^{a}	20.40±3.33^{c}
CML 216	1953	58.00±3.76^{c}	27.28±2.24^{c}
CML 395	355	45.07±1.57^{d}	0.00±0.00^{d}
A04	583	27.44±2.67^{f}	0.00±0.00^{d}
E04	1126	69.45±2.05^{ab}	85.42±3.05^{a}
T04	301	60.47±4.03^{c}	45.60±1.06^{b}
TL21	185	42.16±1.99^{de}	0.00±0.00^{d}
TL22	186	64.52±2.79^{bc}	0.00±0.00^{d}
TL26	166	49.40±.91^{d}	0.0±0.00^{d}
TL27	210	34.28±.88^{ef}	0.0±0.00^{d}

Values are means and their standard errors. Means in each column followed by the same letter are not significantly different according to Tukey's test (P≤0.05).

callus (Figure 1B). The removal of these structures enhanced growth of the nascent callus (Figure 1C) resulting in fully developed (type II) calli after 21 days of culture (Figure 1D). These well-developed calli were later used to initiate cell suspensions.

Primary callus formation was observed in all the genotypes under the current culture conditions of CIM and CMM. The rate of callus induction and friable embryogenic callus formation is outlined in Table 1. The highest frequency of callus induction was recorded in CML 144 at 75.62% and the lowest frequency was observed in A04 at 27.44% (Table 2). Significant differences (P≤0.05) in callus induction were observed among the inbred lines.

Friable embryogenic calli were observed in CML 144, CML 216, E04 and T04 genotypes. The rest of the genotypes gave rise to non-embryogenic callus that showed browning as another salient observation. The number of embryos forming callus were counted and the frequency of callus induction calculated as a percentage of the total embryos cultured. Similarly, calli showing the presence of somatic embryos were counted and frequency of embryogenic calli calculated as a percentage of the total calli transferred to CMM. Frequency of shoot formation was calculated as the total number of shoots formed as a percentage of the total calli transferred to PRM. The highest frequency of friable embryogenic callus was recorded in E04 (85.42%) while the lowest was in CML 144 (20.4%) (Table 2). Incorporation of dicamba and kinetin led to formation of soft and friable callus (Figure 2).

Establishment of cell suspension cultures and determination of packed cell volume

Cell suspension cultures were successfully initiated in two genotypes CML 216 and EO4 using six liquid media regimes designated D1, D2, D3, P1, P2 and P3 (Table 1). Upon determining their packed cell volume (PCV), E04 in media regime D2 showed the highest initial PCV at day 1 with regimes D3 and P2 recording the lowest PCVs (Figure 3). Overall, the highest PCV (average 240 µl) was recorded in media regimes D1 and D2 after 14 days of incubation. On the other hand, media regime D3 recorded the lowest PCV (82 µl) after 21 days (Figure 3). There was an initial increase in the PCV values in most media regimes for the first 7 days in E04. Contrastingly, media regime P1 recorded an initial decrease before increasing from day 7 to day 14 (Figure 3). The levels of change (increase and decrease) varied significantly throughout the time points tested. Inbred line CML 216 had the highest PCV value (188 µl) in media regime D1 on the 7th day. On the other hand, media regime P3 recorded the lowest PCV (68 µl) after day 21. Media regime D1, P2 and P3 showed an initial increase in PCVs with D2, D3 and P1 showing reduced levels within the same time point after day one (Figure 4). Overall, there were significant reductions in PCVs across all the media regimes after day 7 (Figure 4).

A profile of the trend in cell growth in E04 inbred line using media regimes with reduced levels of ammonium nitrate over a period of time is outlined in Figure 5. The PCVs in the 3 treatments and the control decreased until the 4th day of culture with a subsequent increase until day 8 although the increments varied with time (Figure 5). Medium M12 with a 12-fold ammonia reduction recorded the highest PCVs increment than M4 and M8. The control media treatment M1, recorded significantly lower PCV levels as compared to M12 but significantly higher levels than M4 and M8 (Figure 5).

Production of semi-protoplast cultures in tropical inbred lines

Digestion with enzyme regimen containing 2% cellulase produced semi-protoplasts (Figure 6). The enzyme

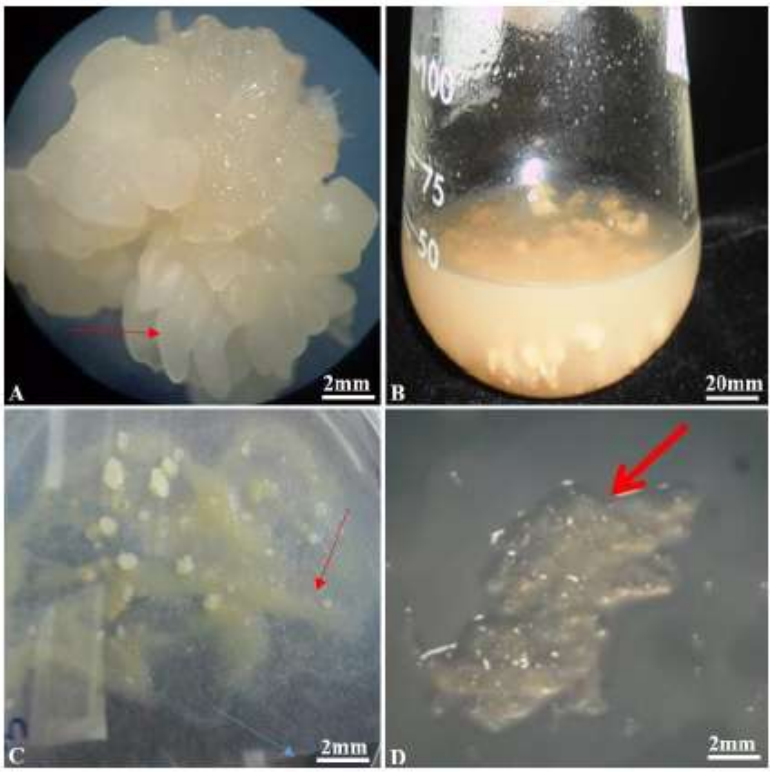

Figure 2. Establishment of cell suspension cultures in tropical maize. (A) Friable embryogenic callus after culture on callus maintenance media. The red arrow shows somatic embryo on the callus. (B) Establishment of cell suspensions in liquid medium in Erlenmeyer flasks. (C) Maize cells and cell clusters growing on Protoplast Regeneration media (PRM) overlaid with a lens paper. The red arrow shows cell cluster starting to form. (D) Cell cluster on solid medium after 21 days of growth in liquid medium (X 40 magnification on a stereo microscope under normal white light).

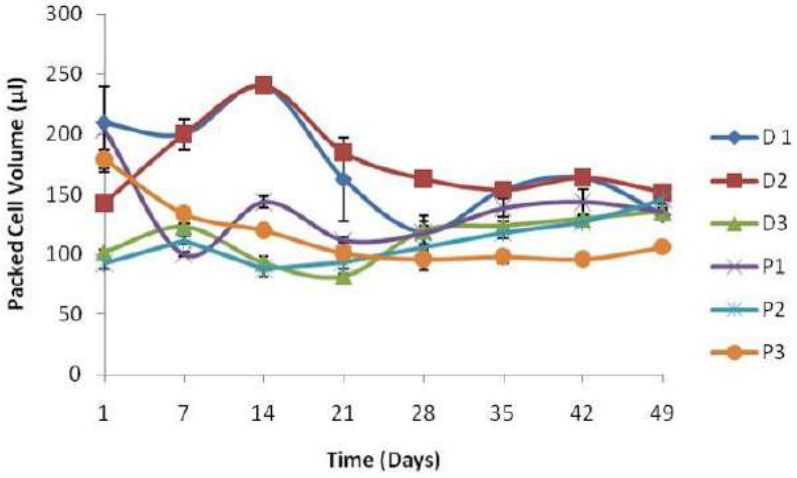

Figure 3. Effect of various media regimes on packed cell volume in cell suspensions of E04: MS + 2 mg/L 2,4-D, 0.8 g/L proline + 0.1 g/L asparagine; D2: MS + 2 mg/L 2,4-D, 0.4 g/L proline + 0.1 g/L asparagine; D3: MS+ 2 mg/L 2,4-D+0.1 g/L asparagine; P1: MS + 3 mg/L picloram, 0.8 g/L proline + 0.1 g/L asparagine; P2: MS + 3 mg/L picloram, 0.4 g/L proline + 0.1 g/L asparagine; P3: MS + 3 mg/L picloram+ 0.1 g/L asparagine.

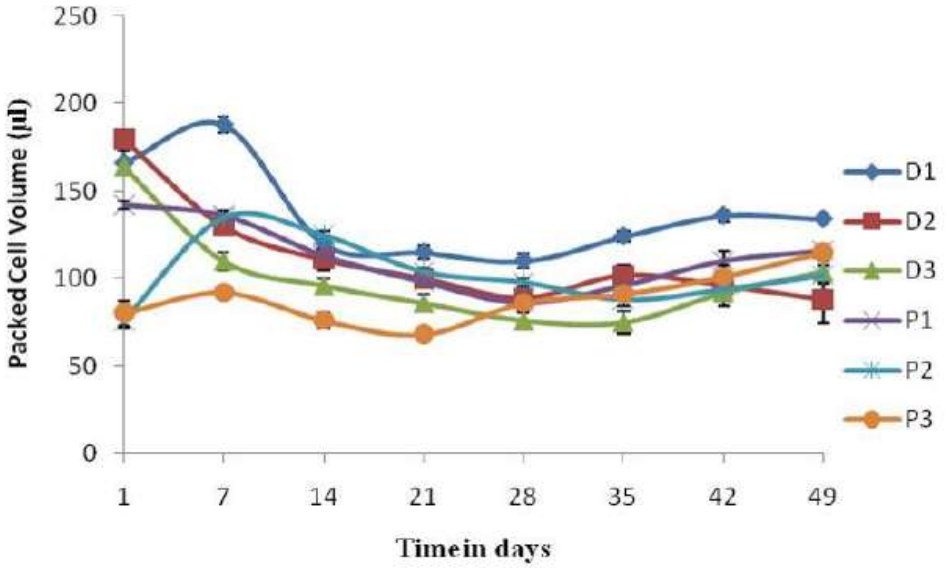

Figure 4. Effect of various media regimes on packed cell volume in CML 216. D1: MS + 2 mg/L 2,4-D, 0.8 g/L proline + 0.1 g/L asparagine; D2: MS + 2 mg/L 2,4-D, 0.4 g/L proline + 0.1 g/L asparagine; D3: MS+ 2 mg/L 211 2,4-D+0.1 g/L asparagine P1: MS + 3 mg/L picloram, 0.8 g/L proline + 0.1 g/L asparagine; P2: MS + 3 mg/L picloram, 0.4 g/L proline + 0.1 g/L asparagine; P3: MS + 3 mg/L picloram + 0.1 g/L asparagine.

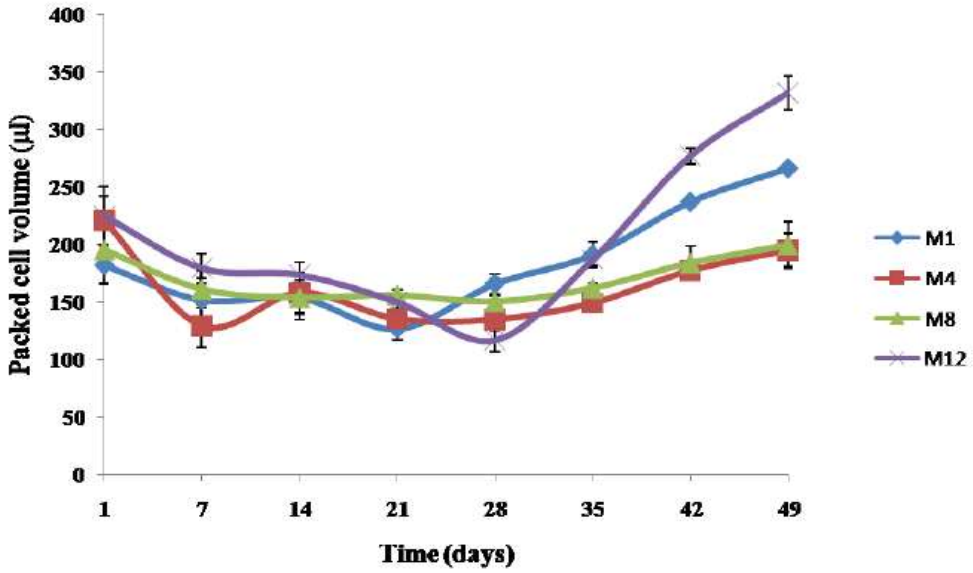

Figure 5. Effect of reduced Concentrations of Ammonium nitrate on packed cell volume (PCV) in EO4. Media M1 had 20.61 mM/L NH_4NO_3 (the normal ammonium concentration in MS used in culture media). The amount of ammonium nitrate reduced in different proportions in M4 (four-fold); M8 (eight-fold); M12 (twelve-fold).

cocktail containing 2% cellulase produced optimal digestion results based on the spherical appearance of the generated protoplasts. Cells treated with digestion regimen containing 1% cellulase yielded low numbers of protoplasts while those digested with enzyme regimen containing 3% cellulase appeared over-digested (Figure 6). Semi-protoplasts produced after digestion with 3% cellulase appeared deformed with characteristic large-

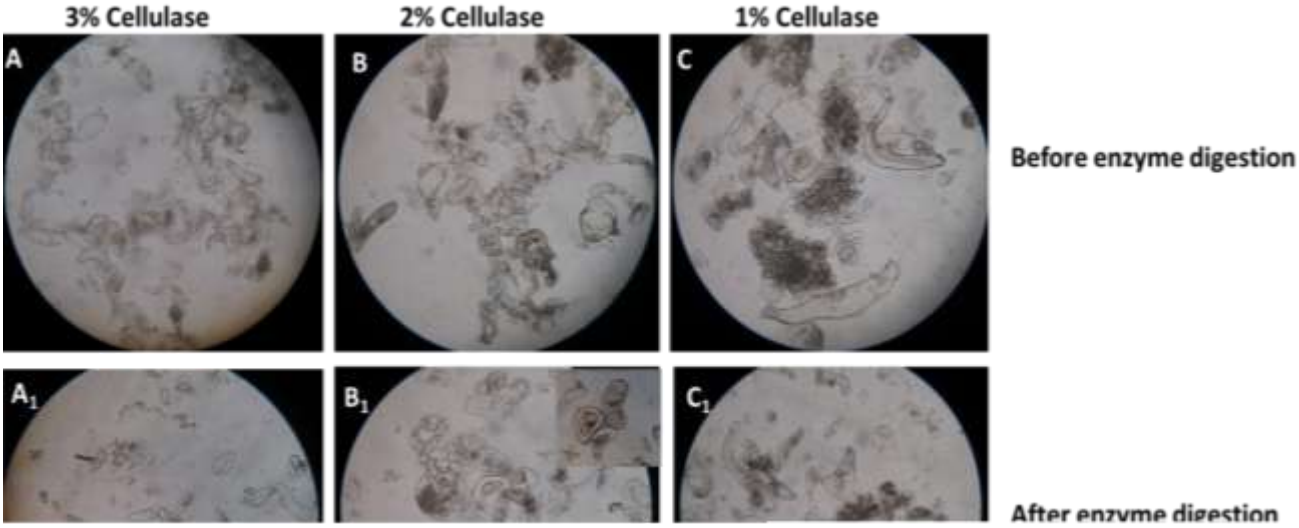

Figure 6. Profiles of Semi-protoplast cells of maize genotype EO4 derived from cell suspensions following digestion using enzymes. The cells are shown before (A, B, C) and 5 hours (A_1, B_1, C_1) of cocktail enzyme digestion. Pictures were taken using a digital camera following observation under a light stereo microscope at X40 magnification and normal white light filter.

Figure 7. Regeneration profile of maize from cell suspension clusters. (A) Cell clusters on Protoplast regeneration media (PRM), (B) Greening callus on CMM showing shoot development. (C) Maize plantlets on regeneration media showing a fully developed shoot with roots. (D) Hardening of plantlets on peat moss in the glasshouse. (E) Acclimatization of the plants in potted soil. (F) Mature plants after the regeneration process.

Table 3. Effect of media regimes on shoot formation and number of shoots per callus in E04 inbred line.

Treatment	Frequency of shoot formation (%)	Average shoots per callus
P1	15.31±3.86[b]	0.81±0.83[a]
P2	28.95±3.45[a]	1.23±0.90[a]

Values are means and their standard errors. Means in each column followed by the same letter are not significantly different according to Turkey's test (P≤0.05). P1: MS + 3 mg/L picloram, 0.8 g/L proline + 0.1 g/L asparagine used in culturing cells used to form callus P2: MS + 3 mg/L picloram, 0.4 g/L proline + 0.1 g/L asparagine used in culturing cells used to form callus.

sized cells; a sign of unsuitable cell types or quality of donor cell suspensions.

Regeneration of whole plants from cell clusters

Callus clusters successfully developed into plantlets while the single cells failed to regenerate. The profile of stages in whole plant regeneration from cell suspensions is summarized in Figure 7. Only genotype E04 was successfully regenerated in this study. The frequency of shoot formation in this genotype varied across media regimes with P2 producing the highest regeneration frequency (28.95%) while P1 recorded a significantly lower frequency (15.31%) (Table 3). In regard to the number of shoots, P2 again produced the highest average shoots per callus (1.23) although this was not significantly different from P1 (0.81) at P≤0.05 (Table 3). On the other hand, callus recovered from cell suspensions maintained on media with 2,4-D did not form shoots. Fully formed plantlets were obtained at the 15th day. The plants were subjected to rooting by being cultured on autoclaved peat moss. These plants acclimatized well on peat moss and were later transferred to large soil filled pots in the open field.

DISCUSSION

In agreement with this study, Anami et al. (2010) used immature embryos to produce friable embryogenic callus. Success is described in the initiation of friable embryogenic calli in selected maize inbred lines, use of these calli to induce cell suspension cultures, and their eventual regeneration into whole plants in maize. Success in the induction of primary callus tissues was achieved in all the genotypes tested using 2, 4-D. However, conversion of these tissues into friable embryogenic structures was observed in only four genotypes, under the current conditions. Conversion of primary callus into friable embryos is genotype dependent, that is why all primary callus did not form friable embryos which is in line with the study by Binott et al. (2008). Only friable embryogenic (Type II) callus cultures can be used in formation of embryogenic cell suspensions for whole plant regeneration (Gordon-Kamm et al., 1990; Songstad, 2010). Genotype dependency in callus induction during *in vitro* maize regeneration has previously been reported (Akoyi et al., 2013; Armstrong et al., 1992). In this study, some of the genotypes formed callus that turned brown, became necrotic, and eventually died which was in line with studies from other tropical maize genotypes (Binott et al., 2008; Ombori et al., 2008).

In maize, type I callus can only be maintained for a short period of time and is therefore unsuitable for generation of suspension cultures which require friable and rapidly growing tissues (Armstrong and Green, 1985; Frame et al., 2000; Vasil and Vasil, 1986). To generate cell suspension cultures, the friable calli need to be cultured in a suitable liquid medium (Dixon, 1985; Gamborg and Phillips, 1995). Under the current study, lack of formation of friable embryogenic calli (FEC) in the six genotypes is attributed to their lack of amenability to tissue culture under the current conditions. Significant differences (P<0.05) in FEC frequencies recorded among the four genotypes confirmed our hypothesis that regeneration of these lines is genotype-dependent and this is in line with a previous study where similar observations were made (Akoyi et al., 2013). Dicamba was instrumental in maintenance of the FEC as this auxin was found to be superior to 2, 4-D. The latter PGR caused precocious germination and produced non-embryogenic calli. Previous studies have also implicated 2,4-D in negative somaclonal variations in regenerants (Akoyi et al., 2013; Omer et al., 2008).

The present study further reports success in establishment of cell suspensions in two genotypes. Continuous agitation of cultures in liquid media was instrumental in obtaining cell suspensions and as expected, cell clusters/clumps were also recovered owing to the often required degree of aggregation (Hulst et al., 1989). Various methods for determination and measurement of growth of cells in culture have been described (Mustafa et al., 2011). Just like initial descriptions (Dixon, 1985; Hahlbrock, 1975), the current study employed the packed cell volume (PCV) technique. Here, growth is determined as the packed cell volume of

pelleted cells and expressed as a function of the volume of culture over time. This has been shown as a fast and simple method for growth measurement of cell suspensions (Mustafa et al., 2011).

The growth of cells in suspension was initially exponential for the first four to eight days before decreasing. This suggests that the factors inhibiting continued exponential growth were effective by the fourth day of culture, becoming more potent thereafter. Alternatively, growth-rate limiting components in the medium were depleted fast by the fourth day of culture leaving the cells without nourishment. A continuous culture system is currently an option for exploration, while the responsible component(s) limiting and/or inhibiting growth are also being identified by optimizing each component of the medium formulation while holding the others constant.

The observed variation in growth of cells in liquid media depended on components incorporated therein. Media containing proline yielded higher levels of PCV than that without confirming the importance of proline in maintenance of friable embryogenic calli for a long time in culture media (Armstrong and Green, 1985; Jones, 2009). Despite the failure of 2, 4 D to produce friable embryogenic callus, it proved to be more superior in yielding higher levels of PCV compared to picloram. Similar studies on effect of auxins on embryogenesis during cell growth in culture have been reported (Ombori et al., 2008). Generally, the use of MS media with 20 mM ammonium nitrate yielded lower PCV values in all the genotypes across media treatments. Reducing these levels, however, resulted in a marked increase in PCV over time. Ammonium nitrate is essential in promoting cell division but is toxic to cells and protoplasts in concentrations used in most tissue culture media. To enhance cell division and reduce toxicity, its concentration needs to be reduced. This is based on reports demonstrating that reducing nitrogen was effective in maintenance of friable embryogenic callus (Jones, 2009). In the current study, a 12-fold reduction of ammonium nitrate resulted in the highest PCV in maize compared to the control. We have also optimized a protocol for generation of tropical maize from semi-protoplasts. An optimum enzyme cocktail for production of semi-protoplasts used in this study was comparable to that used by Armstrong et al. (1990) during production of protoplasts used in PEG-mediated DNA delivery. The slight differences observed could be due to the genotypes studied. The earliest reports on successful maize transformation involved the use of protoplasts to introduce transgenes through microprojectile particle bombardment (Fromm et al., 1990; Gordon-Kamm et al., 1990; Rhodes et al., 1988). Unlike *Agrobacterium*-mediated technique, this method is not host specific (Davey et al., 2000). Protoplasts offer an advantage during transformation, via PEG treatment or electroporation, in that they allow penetration of DNA into the cells with much ease (Schuurink and Louwerse, 2000). These processes, however, require homogeneous cell populations for release of protoplasts and enzyme treatment (Davey et al., 2000; Tyagi et al., 1999).

Genotypes CML 216 and T04 did not regenerate into whole plants despite having formed friable embryogenic calli. This can be attributed to genotype dependence during regeneration in most cereals with similar results being reported in maize regeneration (Ge et al., 2016). Furthermore, lower regeneration efficiencies have been reported for tropical maize inbred lines as compared to temperate ones (Anami et al., 2010; Ombori et al., 2008), thereby necessitating exploration of ways to improve the efficiencies. The media components used during maintenance of the cells in suspension influenced the regenerability of the maize cells and the fact that cells and protoplasts are dedifferentiated and respond less drastically to changes during tissue culture manipulations, has been exploited in this study to obtain plants.

Conclusion

The potential of tropical maize inbred genotypes to induce friable embryogenic callus and sustain growth through cell suspensions has been evaluated. All the maize genotypes tested were capable of inducing callus, although only a few were able to induce friable embryogenic callus that was used to initiate cell suspensions. Embryogenic cells and callus are a prerequisite for maize regeneration and transformation, and their formation is genotype dependent. The embryogenic callus cultures used effectively produced semi-protoplasts and only the cells in clusters were able to produce plants. Therefore, this optimized protocol has the potential for further improvement of these tropical maize genotypes through genetic engineering.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Screening extracts of fungal endophytes isolated from *Allophylus abyssinicus* (Hochst. Radlk.) for control of bean anthracnose

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Common bean is consumed in large quantities globally but it is highly susceptible to *Colletotrichum lindemuthianum*, leading to 90 to 100% yield losses. This study investigated the bioactivity of secondary metabolites from *Allophylus abyssinicus* fungal endophytes against *C. lindemuthianum*, causing bean anthracnose. A total of 37 fungal endophytes were isolated from the leaves, bark and roots of *A. abyssinicus* and 20% identified by ITS-rDNA sequence analysis as *Aspergillus hancockii, Penicillium christenseniae, Penicillium atrosanguineum* and *Penicillium manginii*. Thirty-three of the endophytes were active against *C. lindemuthianum* in the dual culture assay with the highest inhibition being 82.6% (*A. hancockii*). Two of the most active endophytes (*A. hancockii* and *P. christenseniae*) were fermented on rice media and their methanol extracts partitioned between ethyl acetate and hexane. *Aspergillus hancockii* crude hexane extract had the highest inhibition (19.0±1.7mm at 100 mg/ml) against the pathogen under the bioassay screening and 6.3±3.8 mm at 6.25% against *C. lindemuthianum* under the minimum inhibitory concentration (0.625 mg/ml) screening. Chemical screening of the extracts revealed presence of alkaloids in *A. hancockii* and *P. christenseniae*; sterols, triterpenes and coumarins in *A. hancockii*. These results indicated that fungal endophytes from *A. abyssinicus* are a source of active compounds that can be used to control *C. lindemuthianum* affecting common bean.

Key words: *Allophylus abyssinicus*, Antifungal activity, *Colletotrichum lindemuthianum*, fungal endophytes, secondary metabolites.

INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) ranks as the most consumed legume globally contributing to food security and solving malnutrition (Siddiq et al., 2022). It is a source of protein, resistant starch, dietary fibre,

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antioxidants, minerals and vitamins (Aguilera et al., 2011; Pedrosa et al., 2015). Common bean is highly affected by anthracnose caused by *Colletotrichum lindemuthianum* which is seed-borne (Gillard and Ranatunga, 2013) leading up to 100% yield loss in susceptible cultivars (Mohammed, 2013). Disease symptoms occur as dark brown sunken, circular to elliptical lesions that may girdle the stem and on pods. They start as small reddish-brown, slightly sunken circular spots that turn to larger, dark sunken lesions distorting the pods (Jadon et al., 2020). Anthracnose conducive developmental conditions include temperatures of about 17°C, relative humidity above 92% and soil pH of 5.8-6.5 (Padder et al., 2017).

C. lindemuthianum conidium comes into contact with the susceptible bean plant leaf, stem or pod, adheres to the plant cuticle, and germinates (Alkemade et al., 2021). In humid conditions, the conidium germinates and produces an appressorium used for epidermal cell penetration (Jadon et al., 2020). The appressorial surface adhering to the cuticle is flattened and a pore form where the infection peg emerges and pierces the bean leaf cuticle and cell wall. It then penetrates into the host epidermal cell (Chethana et al., 2021). Various enzymes such as cutinase, oxidase and lipases are secreted from the infection peg that degrade plant cuticle and wax layers (Cook and Decuzzi, 2021). The infection peg protrudes from the appressorium, penetrate the cell wall where infection hyphae grow and develop into infection vesicles. *Colletortichum lindemuthianum* is a hemibiotrophic fungus (Dubrulle et al., 2020), spending part of the infection cycle as a biotroph and the other as a necrotrophy. The pathogen establishes an initial asymptomatic biotrophic phase during the infection process, invading its host's tissues (*P. vulgaris*) undetected (Newman and Derbyshire, 2020). Bean anthracnose is a highly destructive disease affecting dry bean production and significantly reduces both seed yield and quality of dry beans under favorable conditions resulting in reduced marketability of the crop.

Various methods have been used to control bean anthracnose such as use of resistant cultivars and chemical control, with the pathogen overcoming the resistance in some commercial cultivars (Rodriguez-Guerra et al., 2003). Biocontrol using beneficial microbes is a much better option compared to chemical control (Medeiros et al., 2012). These beneficial microbes include endophytes which live symbiotically within plant tissues and cause no disease symptoms (Abbamondi et al., 2016). These endophytes inhibit plant diseases by inducing resistance in the host plant, competition for food and space, and also antibiosis (Medeiros et al., 2012).

Allophylus is the largest genus in the Sapindaceae family with about 255 plant species distributed worldwide. Roots and twigs of *Allophylus africanus* are used to treat gastritis, hookworm, venereal diseases, burns, sores and fever in west Africa (Lewis et al., 2003). Roots of *Allophylus ferrugineus* var. *ferrugineus* (Sapindaceae) are used to treat gastritis, coughing, round worms and fever in the Shengena forest reserve of Tanzania (Boer et al., 2005). *Allophylus edulis* is a source of sesquiterpenes (Foster et al., 2004), flavonoids, phenolic compounds (Arisawa et al., 1989), tannins, and essential oils (Rasico, 2007). Roots of *Allophylus abyssinicus* treat coughs and rheumatism in Kenya, while leaves are used to treat helminths and the fruits to cure venereal diseases in Ethiopia (Chavan and Gaikwad, 2013). Powdered leaves of *A. abyssinicus* are applied to treat wounds or ingested to treat inflammatory conditions. This study was undertaken to evaluate the efficacy of secondary metabolites of fungal endophytes from *A. abyssinicus* on *C. lindemuthianum* for potential application in biocontrol of bean anthracnose.

MATERIALS AND METHODS

Plant

Fresh stem bark, root and leaves of *A. abyssinicus* were collected from Mt. Elgon Forest which is tropically humid with a latitude of 1°08˙N, longitude 34° 45˙E and an altitude of 4321 m above sea level. The plant was identified by a plant taxonomist (Professor Samuel T. Kariuki) and voucher specimen was deposited at the Department of Biological Sciences, Egerton University.

Fungal endophyte isolation protocol

Endophytic fungi were isolated from fresh bark, roots and leaves of *A. abyssinicus* using the procedure described by Zinniel et al. (2002) with slight modifications. The samples were cleaned under running tap water and immersed in 70% ethanol for 3 min, followed by immersion in 2.5% sodium hypochlorite solution for 3 min. These samples were rinsed three times (1 min each) with sterile double distilled water, blot dried with sterile paper towels and cut aseptically into sections of approximately 1 mm by 4 mm using sterile scalpel blades. The tissues were plated on PDA media amended with Streptomycin Sulphate (100 mg/l), incubated at 25±2°C for 1-4 weeks and monitored daily for fungal growth. Fungal mycelia of endophyte isolates were sub-cultured on PDA media without antibiotic to obtain pure cultures which were identified using molecular techniques described by Landum et al. (2016).

Dual culture assay of fungal endophytes against *C. lindemuthianum*

Antifungal activity of the fungal endophytes was screened using dual culture assay method as described by Stadler et al. (2004). Two weeks old pure fungal endophytes grown on PDA at 25±2°C were used. Mycelial agar plugs about 7 mm were cut from actively growing cultures and inoculated opposite *C. lindemuthianum* approximately 2 cm apart on PDA plates. The plates were incubated at 25°C for 7-21 days and monitored for growth inhibition. Inhibition zones between the endophyte and phytopathogen (C-T) were measured after 21 days and the resulting percentage inhibition determined as follows:

$$L = \frac{C-T}{C} \times 100\%$$

where L = inhibition of radial mycelia or colony growth; C=radial

growth measurement of pathogen in control; T = radial growth measurement of pathogen in the presence of antagonist (Hajieghrari et al., 2008).

The experiment was carried out in triplicate and Nystatin discs used against *C. lindemuthianum* as the positive control, whereas for the negative control *C. lindemuthianum* was left to grow in absence of the fungal endophyte.

Molecular characterization of fungal endophytes

Molecular characterization of the fungal endophytes was done according to the method described by Landum et al. (2016). The endophytes were grown on PDA plates, genomic DNA extracted from mycelia of the pure cultures and characterization done by sequencing of the ITS region (rDNA ITS).

DNA extraction and amplification

The DNA extraction was done using the BIO BASIC EZ-10 Genomic DNA kit following manufactures instruction. About 60 mg of the fungal mycelia obtained from a 3 to 4-day old culture was added to a 1.5 ml screw cap reaction tube containing approximately 6-10, 1.4 mm Precellys Ceramic Beads. The sample was covered with 600 µl Plant Cell lysis buffer (PCB) (Sodium propionate, sodium cacodylate and BIS-TRIS propane in a ratio of 2:1:2) and homogenized using a homogenizer (Precellys 24 lysis and homogenization, Peq lab, Bertin Technologies). Approximately 12 µl of β mercaptoethanol was added to the sample to aid in protein degradation. The sample was vortexed (IKA MS3 Digital) to mix the components and incubated for 25 min at 65°C in a metal block (MTB 250). Chloroform (600 µl) was added to solubilize the proteins and polysaccharide from the DNA. The sample was then centrifuged (5430 R) at 10,000 rpm for 2 min and the upper layer transferred to a clean Eppendorf tube while the rest was discarded. Approximately 200 µl of Binding buffer was added and the mixture vortexed, followed by addition of 200 µl ethanol which was later vortexed to mix. The mixture was transferred into EZ-10 column placed in a 2 ml collection tube and centrifuged at 12,000 rpm for 1 min. The mixture was centrifuged at 12, 000 rpm and the flow through discarded. Wash solution (500 µl) diluted with ethanol was added to the mixture to remove the salts. The mixture was centrifuged at 12,000 rpm for 1 min and the flow through discarded and the column centrifuged again at 12,000 rpm for 2 min to remove any remaining wash solution. The column was transferred into an empty 1.5 ml Eppendorf tube and 70 µl of TE Buffer, pre-warmed to 60°C, added directly at the center of the EZ membrane to increase the elution efficiency. The sample was incubated for 2 min at room temperature to increase the elution chances of the DNA from the membrane. The tube was centrifuged at 12,000 rpm for 2 min to elute the DNA which was stored at 4°C for further analysis.

To a PCR tube, it was added 0.5 µl of forward primer ITS1F (CTTGGTCATTTAGAGGAAGTAA) and 0.5 µl of reverse primer ITS4 (TCCTCCGCTTATTGATATGC), 12.5 µL of the jump start ready mix containing 20 mM Tris-HCl pH 8.3, 100 mM KCl, 3 mM $MgCl_2$, 0.002% gelatin, 0.4 mM dNTPs (dATP, dCTP, dGTP, dTTP), stabilizers, 0.1 unit/mL Taq DNA polymerase and JumpStart antibody. This was followed by 9.5 µl of distilled water and 2 µl of the template DNA to make a total volume of 25 µl of the mixture per sample. For a negative control, 2 µl of distilled water was used instead of DNA template in the reaction mix. The amplification was done in a thermocycler (Eppendorf® Mastercycler® nexus Thermal Cycler) under the following conditions; initial denaturation of 5 min at 94°C, followed by 34 cycles of denaturation for 30 s at 94°C, annealing at 52°C for 30 s and elongation for 1 min at 72°C. A final elongation of 10 min at 72°C was added. The PCR products were pre-stained with midori green dye and resolved in a 0.8% agarose gel. The visualization was done in a UV transilluminator (Nippon Genetics Europe GMbH) and photographs taken.

PCR product purification and DNA sequencing

The amplified PCR products were purified using BIO-BASIC EZ-10 spin column purification kit following the manufacturer's instructions. To adjust the DNA to the binding conditions, 110 µl of buffer 1 was added to the 22 µl PCR product and mixed thoroughly. The mixed sample were then put on the EZ-10 spin column and incubated for 2 min at room temperature. Later, the sample was centrifuged at 10,000 rpm for 30 s and the flow-through discarded. The DNA was washed by adding 500 µl wash solution, centrifuged at 10,000 rpm for 30 s and the flow-through discarded. The same amount of wash solution was added to the sample and then centrifuged at 10,000 rpm for 1 min and the flow-through discarded. The amplified DNA was eluted in a clean 1.5 ml reaction tube by adding 20 µl of elution buffer pre-warmed to 65°C. The sample was incubated at room temperature for 2 min and then centrifuged at 10,000 rpm for 1 min. The EZ-10 spin column was discarded. The collected DNA was stored at -4°C ready for sequencing.

The amplified DNA was sequenced by Illumina genome analyzer sequencing machine (applied Biosystems 3730 xl DNA analyzer). The forward and reverse primer sequences obtained from the sequencing were aligned by Genious R7 program to get the consensus sequences. The consensus sequences were deposited in NCBI GenBank and compared with those available in GenBank via BLAST searches.

Fermentation of the bioactive fungal endophytes and isolation of their secondary metabolites

Endophytic fungi with high antifungal activity were selected for fermentation and testing of their secondary metabolites. Ninety grams of rice were autoclaved twice at 120°C for 15 min in 500 ml Erlenmeyer flasks containing 90 ml of distilled water per flask. Three agar plugs (2 cm × 2 cm) were cut from seven-day-old endophyte cultures on PDA and used to inoculate each flask. One flask, without inoculum, was used as control. After 21 days of incubation at 25°C, 150 ml of methanol was added to each flask and the contents allowed to stand overnight at room temperature. The methanol was filtered and evaporated under reduced pressure to yield the methanol extract which was then subjected to liquid-liquid partitioning with hexane and ethyl acetate. The resulting organic layer was evaporated under reduced pressure to produce the hexane and ethyl acetate extracts.

Testing for chemical constituents of crude fungal endophyte extracts

The endophyte extracts were tested for steroids, terpenoids, saponins, alkaloids using the methods described by Sofowora (1993). Test for steroids was done using Liebermann-Burchard method where 2 ml of acetic acid was added to 0.2 g of each extract and the solution cooled in ice, followed by the addition of conc. H_2SO_4 carefully. Colour development from violet to blue or bluish-green indicated the presence of a steroidal ring that is aglycone portion of cardiac glycoside, while colour change from pink to violet indicated presence of terpenoids whereby the extract was dissolved in ethanol followed by addition of 1 ml of acetic anhydride and conc. H_2SO_4. One gram of each extract was boiled in 5 ml of distilled water and filtered; 3 ml of distilled water was added to the filtrate and shaken vigorously. Formation of Froth indicated

presence of saponins (Sofowora, 1993). Test for flavonoids was done using Shinoda's test (Trease and Evans, 2002) whereby the extracts were dissolved in 1.5 ml of 2% hydrochloric acid followed by addition of 2-3 drops of Mayers reagent. Alkaloids were detected by the formation of a creamy or white precipitate (Sofowora, 1993). Three mililitres of 10% NaOH was added to 2 ml of the different extracts dissolved in distilled water and a change in colour from the original to yellow indicated presence of coumarins (Velavan, 2015). Presence of sterols and triterpenes was tested using Liebermann-Burchard Reaction (Velavan, 2015) whereas that of anthraquinones was done using Borntrager's Test (Sofowora, 1993). Two milliliters of the extracts dissolved in distilled water were added to 2 ml of 2 N hydrochloric acid and ammonia, and formation of a pink red colour that turns blue-violet indicated the presence of anthocyanins (Velavan, 2015). About 10 ml of distilled water was added to 0.5 g of the extract stirred, filtered and 2-3 drops of 1% ferric chloride solution added to the filtrate and formation of a blue-black, green or blue-green precipitate indicated the presence of tannins (Trease and Evans, 2002). Emodins, on the other hand, were tested by adding 2 ml of NH_4OH and 3 ml of Benzene to the extract and their presence detected by formation of a red colour (Savithramma, 2011). Leucoanthocyanins were screened by addition of 5 ml of the aqueous extract to 5 ml of isoamyl alcohol leading to the appearance of red colour in the upper layer.

Bioassay of fungal endophyte crude extracts against *C. lindemuthianum*

Fungal endophytes extracts were subjected to qualitative secondary screening for their antifungal activities using the disc diffusion method with slight modifications, as described by Premlata et al. (2012). Five Agar blocks were cut from actively growing *C. lindemuthianum* culture and transferred into a sterile vial containing 5 ml of sterilized distilled water. The culture blocks were crushed using a sterile glass rod to make a fungal suspension. One milliliter of the fungal suspension was uniformly spread on sterile PDA media in Petri dishes using a sterile cotton swab. From the dried methanol, hexane and ethyl acetate extracts 20 mg was weighed and dissolved in 1ml of DMSO to make a concentration of 20 mg/ml. Sterilized 6 mm blank sensitivity discs were impregnated with 10 µl of the 20 mg/ml concentration of the different extracts and placed at equal distance on the inoculated culture plates. The plates were incubated at 25°C for 7-21 days and inhibition zone diameters measured. The experiments were done in triplicate; Nystatin was used as a positive control and DMSO as the negative control.

Minimum inhibitory concentration (MIC) of bioactive fungal endophyte crude extracts

MIC for the bioactive extracts was determined by testing double fold serial dilutions following the method used by Agbabiaka and Sule (2008) with slight modifications. Methanol extract was not subjected to MIC test due to low inhibition against the test pathogen. Initial concentration of 200 mg/ml (stock solution) was prepared by measuring 400 mg of the hexane and ethyl acetate extracts and dissolving in 2 ml of DMSO. By diluting 1 ml of 200 mg/ml (stock solution) to 2 ml using 1 ml of DMSO, 100 mg/ml concentration was obtained. From 100 mg/ml (stock solution), 1 ml was diluted to 2 ml using 1 ml of DMSO to make a concentration of 50 mg/ml. The aforementioned process was repeated several times to obtain the other concentrations of 25, 12.5 and 6.25 mg/ml. Then 6 mm diameter sterile sensitivity discs were impregnated with 10 µl of the different concentrations and placed at different points on *C. lindemuthianum* inoculated culture plates. After allowing the sample to diffuse for 5 min, the plates were incubated at 25°C for 7 to 21 days. The experiments were done in triplicates; Nystatin was used as a positive control and DMSO as the negative control. Inhibition zone diameters between the pathogen and extracts were measured after 21 days and the averages determined.

RESULTS

Isolation of fungal endophytes and antifungal screening

A total of 37 different fungal endophytes were isolated from the leaves, stem bark and roots of *A. abyssinicus.* Nine of the endophytes (24.3%) were isolated from the leaves, 23 from the bark (62.2%) and five from the roots (13.5%), indicating that a larger percentage of the fungal endophytes inhabit the upper parts of the host plant. Dual culture assay showed that 33 out of 37 fungal endophytes had antagonistic activity against C. lindemuthianum (Figure 1, Table 1). Endophytic fungus *A. hancockii* (Aal - 20) had the highest percentage inhibition zone of 82.6±6.5 against *C. lindemuthianum* followed by Aar-9 (79.7±3.3), Aal-19 (71.7±11.5), and Aar-25 (71.3±1.6). The activity of fungal endophyte Aab-14 and Aar-23 against C. lindemuthianum had no significant difference ($P > 0.05$) as compared to nystatin standard while Aab-36 had a low percentage inhibition of 4.0±3.5 against the test pathogen. Fungal endophytes isolated from the roots of *A. abyssinicus* had the highest percentage inhibition of 58% on average against the test pathogen followed by fungal endophytes isolated from the leaves at 51% and those from the bark having average percentage inhibition of 31.2%.

Molecular identification of fungal endophytes

Four fungal endophytes that showed good antifungal activity against *C. lindemuthianum* were identified using the ITS-rDNA sequence analysis. They included *Aspergillus hancockii, Penicillium christenseniae, Penicillium atrosanguineum* and *Penicillium manginii* (Table 2). Optimal PCR products of the four bioactive isolates were obtained using primer pair ITS1F and ITS4 and the BLAST percentage similarity of the identified fungal endophytes to the sequences in NCBI from the previously identified fungi ranged from 99.66 to 100%.

Bioassay and MIC of endophytes' extracts

Extracts from the isolated fungal endophytes showed varying activity against *C. lindemuthianum* (Table 3) except the crude methanol extract of *P. christenseniae*. *A. hancockii* hexane crude extract gave the highest inhibition of 19.0±1.7 mm at 100% concentration (100 mg/ml) against the test pathogen compared to the Nystatin standard at 48.0±3.5 mm. The ethyl acetate

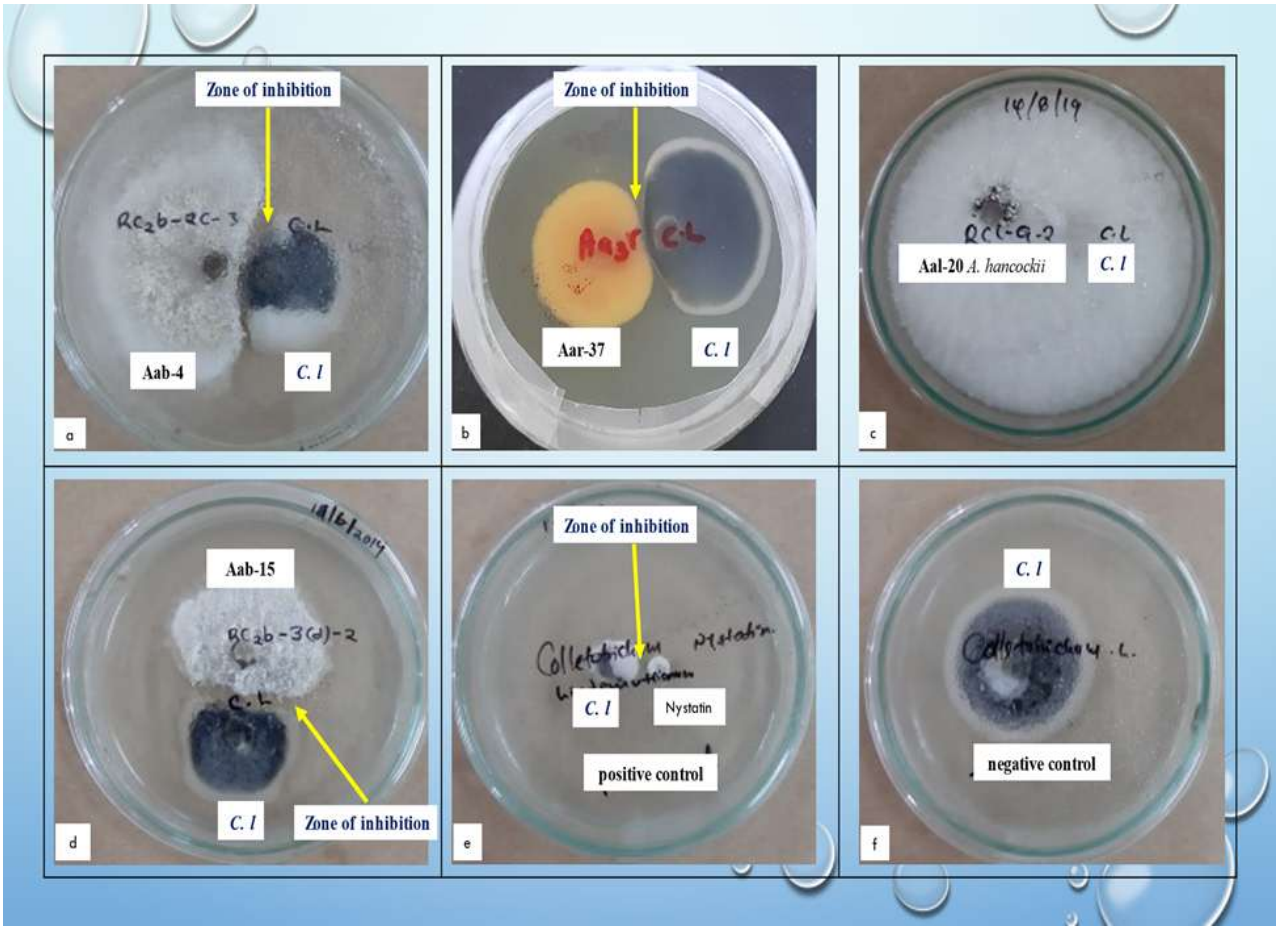


Figure 1. Dual culture test results (a-d) of selected endophytic fungi (Aab-4, Aar-37, Aal-20 and Aab-15) from *Allophyllus abyssinicus* against *Colletotrichum lindemuthianum* (*C. l*), e – positive control using nystatin against the pathogen and f – negative control with the pathogen left to grow on its own.

crude extract of *A. hancockii* had a similar inhibition (8.0±1.7 mm) as its crude methanol extract (8.0±1.0 mm) but both exhibited a lower inhibition compared to Nystatin. Both hexane (8.3±0.6 mm) and ethyl acetate (10.3±0.6 mm) crude extracts of *P. christenseniae* had some inhibition against *C. lindemuthianum* which was lower than Nystatin. Hexane extracts from both fungal endophytes had a good inhibition against the test pathogen, with the methanol extract of *P. christenseniae* lacking activity. The activity of *P. christenseniae* and *A. hancockii* extracts corresponds to the data obtained in the dual culture assay, with *A. hancockii* having higher inhibition.

A. hancockii hexane extract had the highest inhibition of 19.0±1.7 mm (100% concentration) compared to all the other extracts from the two fungal endophytes with *A. hancockii* ethyl acetate crude extract having the lowest inhibition of 8.0±1.7 mm (Table 4). These inhibition results of *A. hancockii* are in line with both the dual culture and bioassay screening of its hexane extracts against *C. lindemuthianum.* On average, *P. christenseniae* gave a higher inhibition against the test pathogen compared to *A. hancockii* at lower concentrations, with both fungal endophytes having lower inhibition compared to the standard Nystatin. *P. christenseniae* hexane extract had a lower inhibition of 8.3±0.6 mm compared to its ethyl acetate extract at 10.3±0.6 mm. Minimum inhibitory concentration for the extracts was determined as 0.0625 mg/ml ml and 0.313 mg/ml for both *A. hancockii* and *P. christenseniae* hexane and ethyl acetate crude extracts respectively. At 6.25% concentration, *A. hancockii* hexane extracts gave inhibitions of 6.3±3.8, while *P. christenseniae* gave inhibitions of 8.0±2.0 and 7.3±1.5 mm (hexane and ethyl acetate extracts, respectively).

Chemical constituents in crude extracts of *Allophyllus abyssinicus* fungal endophytes

Steroids were only present in *P. christenseniae* methanol and ethyl acetate crude extracts but absent in *A.*

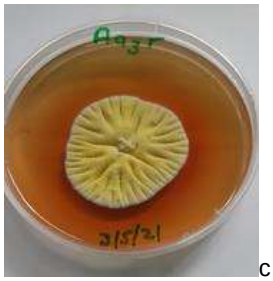
Table 1. Inhibition of different *Allophyllus abyssinicus* fungal endophytes against *Colletotrichum lindemuthianum*.

Fungal endophyte	Percentage inhibition (n=3)
	C. lindemuthianum
Aab-1	20.1±3.4^{ab}
Aab-2	25.4±3.4^{abc}
Aab-3	60.1±9.0^{fghi}
Aab-4	38.4±8.2^{bcd}
Aal-5	40.2±4.8^{bcdef}
Aal-6	34.1±2.5^{bcd}
Aab-7	22.5±4.5^{ab}
Aal-8	44.9±2.5^{cdef}
Aar-9 (*P. christenseniae)*	79.7±3.3^{ij}
Aal-10	43.5±13.2^{cdef}
Aab-11	10.9±5.8^{a}
Aab-12	26.8±2.5^{abc}
Aab-13	36.9±2.0^{bcd}
Aab-14	65.2±9.5^{ghij}
Aab-15	33.3±5.0^{bcd}
Aal-16	59.4±5.5^{efgh}
Aal-17	39.9±7.0^{bcde}
Aal-18	47.8±5.8^{defg}
Aal-19	71.7±11.5^{hij}
Aal-20 (*A. hancockii*)	82.6±6.5^{j}
Aab-21	62.2±22.3^{ghi}
Aab-22	63.9±2.8^{ghi}
Aar-23 *(P. manginii)*	66.7±5.6^{ghij}
Aab-24	59.3±4.2^{efgh}
Aar-25 *(P. atrosanguineum)*	71.3±1.6^{hij}
Aar-26	44.1±10.9^{cdef}
Aab-27	37.4±12^{bcd}
Aab-28	42.6±5.8^{cdef}
Aab-29	35.9±7.6^{bcd}
Aab-30	6.0±2.5^{a}
Aab-31	27.3±14.6^{abc}
Aab-32	6.0±1.2^{a}
Aab-33	6.0±3.3^{a}
Aab-34	47.7±17.6^{defg}
Aab-35	6.0±2.7^{a}
Aab-36	4.0±3.5^{a}
Aar-37	31.3±7.5^{bcd}
NYSTATIN	63.5±1.8^{ghij}

Within a column, fungal endophytes sharing the same letter(s) are not significantly different in antagonism against *Colletotrichum lindemuthianum* while those with different letters are significantly different (α =0.05, Tukey's test). The inhibition zone values are the mean of three replicates ±S.D. of the mean. Aal = *A. abyssinicus* leaves; Aar =*A. abyssinicuss* roots; Aab =*A. abyssinicus* bark.

hancockii. Saponins and alkaloids were present in both endophyte extracts, while flavonoids were found only in *A. hancockii* methanol and hexane extract. Coumarins, sterols and triterpenes were also only present in *A. hancockii* whereas Leucoanthocyanins were only found in *P. christenseniae.* From the chemical constituent screening of these two fungal endophytes extracts there were no traces of terpenoids, anthraquinones,

Table 2. Identification of fungal endophytes from *Allophylus abyssinicus* using molecular techniques.

Fungal endophyte isolate	Molecular identification		
	Similarity %	Accession number	Similarity with
 a	99.6	MT530175.1	*P. christenseniae* (Aar - 9)
 b	99.66	NR154725.1	*A. hancockii* (Aal - 20)
 c	100	MH857072.1	*P. atrosanguineum* (Aar - 25)
 d	100	MH858641.1	*P. manginii* (Aar - 23)

anthocyanins, tanins, and emodins (Table 5).

DISCUSSION

Isolation and antifungal activity of *A. abyssinicus* fungal endophytes and their extracts

Different fungal endophytes were isolated from the medicinal plant *A. abyssinicus* fresh stem bark, roots and leaves. This isolation results are in agreement with the study by Carbungco et al. (2017) whereby 24 different fungal endophytes were isolated from Moringa oleifera leaves indicating that different fungal endophytes can be isolated from different parts of a plant. The results also confirm that endophytes are present in all plant tissues of the host plant and are rich sources of active biomolecules as reported by Kusari et al. (2012). Very little research has been done on isolation and diversity of endophytes from *A. abyssinicus* but recently *Moniliophthora*

Table 3. Inhibition zones of selected bioactive fungal endophyte crude extracts against *Colletotrichum lindemuthianum*.

Crude fungal endophyte extract	Inhibition diameter in mm (n=3) Bioassay
A. hancockii MeoH	8.0±1.0[a]
A. hancockii Hexane	19.0±1.7[b]
A. hancockii EtoAc	8.0±1.7[a]
P. christenseniae MeoH	6.0±0.0[a]
P. christenseniae Hexane	8.3±0.6[ab]
P. christenseniae EtoAc	10.3±0.6[ab]
Nystatin	48.0±3.5[c]

Within a column, fungal endophytes extracts sharing the same letter(s) are not significantly different in inhibition against *Colletotrichum lindemuthianum* while those with different letters are significantly different (α =0.05, Tukey's test). The inhibition zone values are the mean of the triplicates ±S.D. of the mean. MeoH: Methanol and EtoAc: ethyl acetate.

Table 4. Inhibition zones and MIC of selected bioactive fungal endophyte crude extracts against *Colletotrichum lindemuthianum*

	100%	50%	25%	12.5%	6.25%	3.125%	(MIC mg/ml) 0.625%
Nystatin		+ve=64					-ve=0
A. hancockii							
Hexane	19.0±1.7	10.3±3.5	8.0±2.6	6.7±0.7	6.3±3.8	6.0±0.5	0.6256
EtoAc	8.0±1.7	6.7±0.6	6.0±3.5	6.0±3.5	6.0±0.0	6.0±0.0	5.0006
P. christenseniae							
Hexane	8.3±0.6	8.0±2.0	8.0±1.0	8.0±1.5	8.0±2.0	6.8±1.5	0.313
EtoAc	10.3±0.0	8.7±1.5	8.0±1.7	7.7±2.1	7.3±1.5	6.2±1.9	0.313

perniciosa a non-pathogenic endophytic biotype was isolated from the bark of *A. edulis* in research by Lisboa et al. (2020). Most of the fungal endophytes isolated from *A. abyssinicuss* were situated above the ground with 9 from the leaves, 23 from the stem bark compared to those isolated from the roots which were 5 and all had different characteristics. Results obtained from this isolation are also in agreement with those reported by Alvin et al. (2014) where every plant species hosts various endophytes depending on their habitat and different plant parts.

Different fungal endophytes isolated from *A. abyssinicus* exhibited varying activity against *C. lindemuthianum* in the dual culture assay, with *A. hancockii* isolated from the leaves having the highest percentage inhibition (82.6±6.5) against the test phytopathogen. It is worth noting that fungal endophytes isolated from the roots had the highest percentage inhibition (58%) on average against the test phytopathogen followed by those from the leaves (51%) and bark at (31.2%). *P. christenseniae* isolated from the roots had the second highest percentage inhibition (79.7±3.3). This result corresponds to Korejo et al. (2014) findings where endophytic *Penicillium* species exhibited significant antifungal activity against *Fusarium solani, Fusarium oxysporum* and *Rhizoctonia solani*. *Penicillium christenseniae* SD.84 isolated from the soil had antimicrobial activity against several bacterial and fungal pathogens *in vitro* as reported by Wang et al. (2022). Both *A. hancockii* and *P. christenseniae* have not been previously screened against *C. lindemuthianum*.

Crude extracts from the two most active fungal endophytes, *P. christenseniae* and *A. hancockii* exhibited antifungal activity against the test pathogen in the bioassay tests. The fungal endophyte *P. christenseniae* hexane extract having an inhibition of 8.3±0.6 100 mg/ml concentration against *C. lindemuthianum* results are in line with the findings of Du Toit (2020) that reported *Penicillium* isolates from the tubers of *Pelargonium sidoides* having antifungal activity against *F. oxysporum* f. sp. *cubense. Aspergillus hancockii* hexane extract had an of inhibition of 19.0±1.7 mm (100 mg/ml) against *C. lindemuthianum* which is in agreement with the findings of Hashem et al. (2022) that reported the endophytic *Aspergillus terreus* isolated from *M. oleifera* leaves to have antifungal activity against *Rhizopus oryzae*, *Mucor*

Table 5. Chemical constituents of crude fungal endophytes' extracts from *Allophyllus abyssinicus*.

Chemical constituent	*A. hancockii* extract			*P. christenseniae* extract		
	Meoh	Hexane	EtoAc	Meoh	Hexane	EtoAc
Steroids	-	-	-	+	-	+
Terpenoids	-	-	-	-	-	-
Saponins	+	+	+	+	-	+
Flavonoids	+	+	-	-	-	-
Alkaloids	+	+	+	+	+	-
Coumarins	+	-	+	-	-	-
Sterols and tri terpenes	+	+	+	-	-	-
Anthraquinones	-	-	-	-	-	-
Anthocyanins	-	-	-	-	-	-
Tanins	-	-	-	-	-	-
Emodins	-	-	-	-	-	-
Leucoanthocyanins	-	-	-	+	-	+

racemosus and *Syncephalastrum racemosum*, with its ethyl acetate crude extracts (10 mg/ml) inhibitions being 20, 37, and 18 mm, respectively. The ethyl acetate and hexane crude extracts of the two endophytes were active against *C. lindemuthianum* and their mean inhibitions were statistically different compared to the Nystatin standard, with *A. hancockii* hexane crude extract being statistically lower than Nystatin and *P. christenseniae* hexane and ethyl acetate crude extracts being statistically lower than the Nystatin mean inhibition.

Antifungal activity of *A*. *hanckockii* against the test pathogen is in line with the results obtained by Nirmal et al. (2018) whereby compounds isolated from *Aspergillus banksianus* had antimicrobial activities and since *A*. *hanckockii* belongs to the same genus as *A. banksianus*. Bolaamphiphilic antifungal compounds, the burnettramic acids were isolated from *Aspergillus burnettii* in research by Li et al. (2019); this antifungal activity has similarity as those of *A. hancockii* against *C. lindemuthianum*. In another study, compounds isolated from *A. burnettii* had antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Candida albicans* and *Saccharomyces cerevisiae* in the bioassay screening (Gilchrist et al., 2020) indicating that extracts from fungal endophytes in the genus *Aspergillus* have antimicrobial properties. Antifungal activity of *P*. *christenseniae* is in line with the findings of Wang et al. (2022) indicated that secondary metabolites isolated from this fungus had antimicrobial activity against *S. aureus* and methicillin-resistant *S. aureus*.

MIC of *A. hancockii* were determined as 0.625 mg/ml, 5.000mg/ml and gave inhibitions of 6.3±3.8 mm and 6.7±0.6 mm for the hexane and ethyl acetate extracts, respectively against the test pathogen. At MIC of 0.313 mg/ml *P. christenseniae* hexane extract gave an inhibition of 6.8±1.5 mm. *A. hancockii* hexane extract gave the highest inhibition of 19.0±1.7mm at 100% concentration compared to all the other extracts from the two fungal endophytes with the lowest inhibition obtained from *A. hancockii* ethyl acetate crude extract at 6.0±0.0 mm at 6.25% concentration. This result indicates that the inhibition results of *A. hancockii* are in line with both the dual culture and bioassay screening of its hexane extracts against *C. lindemuthianum.* On average *P. christenseniae* gave a higher inhibition against the test pathogen compared to *A. hancockii* under lower concentrations 6.25%, with both fungal endophytes having very low inhibition compared to the standard Nystatin at 64 mm. Under lower concentrations 6.25% *P. christenseniae* hexane extract gave a higher inhibition diameter of 8.0±2.0 mm compared to its ethyl acetate extract at 7.3±1.5 mm. Results obtained in this study are in line with the findings of Intaraudom et al. (2013) whereby picolinic acid derivatives isolated from a *Penicillium* spp*.* had antifungal activity at MIC values of 1.5 and 3.7 µg/ml. Hexane extracts from both fungal endophytes gave higher inhibition against the pathogens compared to their ethyl acetate extracts. This study, therefore, demonstrates that extracts from the two fungal endophytes isolated from *A. abyssinicus* can be used in agriculture to control common bean anthracnose.

Identification of the fungal endophytes

Out of the 37 fungal endophytes isolated, four that were highly active against *C. lindemuthianum* in the dual culture assay were identified using molecular techniques and they all belonged to the phylum Ascomycota. These results are in agreement with a study by Kirk et al. (2008) that reported Ascomycota as the largest phylum under kingdom Fungi with over 64,000 species and referred to as sac fungi with septate hyphae. Diversity of these fungal endophytes in various plants is normally influenced

by environmental factors, the host and its genetic constitution according to Chen et al. (2010) which is in line with the results obtained in this study whereby different endophytes were isolated from different parts. A lot of endophytes are isolated from this phylum due to their ability to produce ascospores that confer resistance against other microbes under unfavorable environments (Goveas et al., 2011); this is in agreement with the identification results obtained where the bioactive endophytes identified were Ascomycetes.

Three of the isolated active fungal endophytes belonged to the genus *Penicillium* while one belonged to the genus *Aspergillus*. A study by Visagie et al. (2014) indicated *Penicillium* as one of the largest genera in kingdom Fungi, with more than 400 species, distributed globally which is supportive of the results obtained in this study whereby three of the identified endophytes were *Penicillium* spp. *A. hanckockii* isolated in this study has also been previously isolated from soil in peanut fields and other substrates in southeast Australia according to Pitt et al. (2017). The fungus *P. christenseniae* isolated in this research was also isolated from meat products and spices as *P. christenseniae* isolate E20408 in research done by Abd El-Tawab et al. (2020), while *Penicillium atrosanguineum* was isolated by Korur et al. (2019) from forest soil using Waksman's 'Soil Dilution Method' in the study of diversity of *Penicillium* spp. *Penicillium manginii* isolated in this study was isolated as an endophytic fungus (*P. manginii* YIM PH30375), living inside the elder root of *Panax notoginseng* medicinal plant in China in a study by Pei et al. (2015).

Screening of chemical constituents of the fungal endophyte extracts

Various secondary metabolites were present in different fungal endophytes isolated from *A. abyssinicus* in this study. These included; steroids, saponins, flavonoids, alkaloids, coumarins, sterols, tri-terpenes and leucoanthocyanins. The genus *Allophylus* contains medicinally important phytochemicals such as phenols, alkaloids, saponins, sesquiterpenes, steroids, anthraquinones among others isolated from the different plant parts and its endophytes. Presence of steroids, saponins, alkaloids and leucoanthocyanins in *P. christenseniae* is in agreement with a study done by Domracheva et al. (2021) which indicate that *Penicillium* spp. contains active natural products and this secondary metabolites are involved in inhibitory activities against microbes (Kozlovskii et al., 2013). Presence of alkaloids in *P. christenseniae* crude extracts is in line with the findings of Wang et al. (2022) whereby, two new quinolone alkaloid enantiomers, (*Ra*)-(-)-viridicatol (1) and (*Sa*)-(+)-viridicatol (4) and seven other known compounds were isolated from *P. christenseniae* SD.84. The presence of steroids in *P. christenseniae* ethyl acetate crude extract could have contributed to the inhibition of 10.3±0.6 mm, with the difference in inhibition being probably a result of synergy of the chemical constituents present in each extract. This is in line with the report of Chavan and Gaikwad (2016) that indicates the presence of steroids in *A. edulis* which is in the same genus as *A. abyssinicus*.

The presence of flavonoids in *A. hancockii* hexane extract could have contributed to the high inhibition of 19.0±1.7 mm compared to the other extracts. The presence of coumarins, sterols and triterpenes in *A. hancockii* could be the reason why this fungal endophyte was very active in the dual culture, extract bioassay and minimum inhibitory concentration tests against the test pathogen. A study by Chavan and Gaikwad (2016) reported the presence of flavonoids, coumarins, sterols and triterpenes in *A. edulis* which were also present in *A. hancockii* and could have produced a synergistic effect that increased inhibition of *C. lindemuthianum in vitro* since these compounds are absent in *P. christenseniae* which was less active compared to *A. hancockii.* The fungal endophyte *A. hancockii* was reported to yield a total of 69 different types of secondary metabolites, one of them being a novel metabolite called dehydroterrestric acid in a study by Pitt et al. (2017); others isolated included Kojic acid, Onychocin A and B, Speradine F, 7-hydroxytrichothecolon among others which are in line with the findings of this research that various compounds can be isolated from a specific endophyte. A combination of all these different metabolites produced by this fungal endophyte may confer antifungal activity against *C. lindemuthianum* suggesting that they may be toxic or lethal to the pathogen and can be used in management of bean anthracnose.

Results from this study indicate that bioactive secondary metabolites from *A. abyssinicuss* fungal endophytes can be an effective alternative in the control of bean anthracnose due to the fact that biocontrol pose no dangers to the environment and human beings. It is cheaper, compatible with other disease management methods and are highly biodegradable. Use of this secondary metabolite in the disease control will lead to increased production of beans of high quality, thereby increasing their marketability, contribute in reducing food insecurity and hidden hunger to the ever-increasing human population.

Conclusion

This study proved that the medicinal plant *A. abyssinicus* hosts different strains of endophytic fungi that can be used effectively to control *C. lindemuthianum*. The data obtained indicated that these fungal endophytes contain secondary metabolites with antifungal activity against *C. lindemuthianum* and, therefore, can be used to manage

bean anthracnose and boost bean production globally.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Quality evaluation of low free fatty acid and high free fatty acid crude palm oil and variation of total fatty matter and fatty acid composition in Nigerian palm oil

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Quality of palm oil determines its consumer and market acceptability and price. This correlates directly to its moisture (%), free fatty acid (% FFA) and total fatty matter (%TFM) content. The aim of the present study was to evaluate the quality of low free fatty acid (LFFA) and high free fatty acid (HFFA) crude palm oil (CPO) samples aged 5 to 10 days, purchased from four locations in Southern Nigeria. HFFA CPO is produced using traditional methods while LFFA CPO is produced by modern milling methods. The oil samples were analyzed for quality and fatty acid using standard analytical methods. The results obtained showed that FFA and total contaminants were significantly (p<0.05) lower in LFFA and higher in HFFA CPO samples. The values of FFA were significantly (P<0.05) higher in HFFA (9.25±0.70-12.76±1.20%) when compared to LFFA CPO values (2.44±0.30-2.95±0.08%). No significant (p>05) difference was observed in the mean saponification value of LFFA (198.95±0.80 mg KOH/g oil) and HFFA CPO (198.62±0.40 mg KOH/g oil). TFM for LFFA CPO ranged between 91.94±0.40 - 92.45±0.75% suggesting no significant (p>0.05) variability in TFM values for LFFA CPO. TFM values for HFFA CPO were significantly (P<0.05) lower and varied between 81.06±0.64 and 85.16±1.05%. The palmitic acid in HFFA CPO was 44.670±0.85 and 45.641±1.77% in LFFA CPO. Oleic acid content was 37.370±0.92% in HFFA oil and 39.005±1.06% in LFFA oil. It was concluded that CPO is rich in SFAs, MUFAs and PUFAs. The ratio of TSFAs to TUFAs for both LFFA and HFFA CPO is 1:1.

Key words: Quality, crude palm oil, free fatty acid, total fatty matter, fatty acids, gas chromatography-mass spectrometry (GC-MS).

INTRODUCTION

The increasing demand for cooking oil and bio-fuels has made crude palm oil (CPO) the dominant globally traded vegetable oil (Baterman et al., 2010). In Nigeria, CPO is extracted from the fruit pulp of palm fruits (*Elaeis guineensis)* using either traditional or modern milling methods. The plantation varieties of oil palms are different from the wild varieties grown by traditional farmers in Nigeria. And the CPO extracted by modern

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methods and improved technologies (LFFA CPO) is different in quality from the traditional method of extraction based on cultural practices (HFFA CPO). The fresh fruit bunch (FFB) of the wild varieties is usually larger in size than the plantation varieties. In Nigeria, fruits from the wild varieties contain bigger kernel and less pulp (mesocarp) compared to the plantation variety with relatively smaller kernel and fleshy pulp. The composition of CPO includes triacylglycerols (TAGs), diacylglycerols (DAGs) and monoacylglycerols (MAGs) and free fatty acids (FFAs) which are the major components (95-99%), while the minor components (1-5%) consist of sterols, carotenoids, tocopherols, aliphatic alcohols, gums and phosphatides (Prasanth and Gopala, 2014).

Natural fats and oils vary widely in their physico-chemical properties, even though they are made up of the same constituents including fatty acid composition. This is because individual fats and oils vary over relatively large ranges in the proportion of the component fatty acids, and the structures of the individual component triglycerides (Sonntag, 1979). Although these two factors are interdependent, there are aspects of the overall effects that can more or less be attributed to one or the other. This is the case with CPO. The quality of CPO varies from one source to the other depending on several factors including age of fruits, age of oil, processing method, handling and storage conditions (Sonntag, 1979). A wide variation in the quality of locally extracted CPO (HFFA grade) sold in Nigerian markets in Lagos (Adebayo-Oyetoro et al., 2019) and Port Harcourt (Ohimain et al., 2012) has been reported.

Many hitherto unappreciated factors are now known to influence the fatty acid composition as well as the triglyceride structure of natural fats and oils. Among the factors which affect the composition of fats and oils in the vegetable kingdom are climatic conditions, soil type in which the parent plant was grown, geography of the growing location, hydrology of the area, maturity of the plant, health of the plant, environmental conditions, processing culture and traditions, handling and storage conditions and most importantly, genetic variations in the plant (Sonntag, 1979). For instance, the plantation varieties of oil palms are different from the wild varieties grown by traditional farmers in Nigeria. And the CPO extracted by modern methods and improved technologies (LFFA CPO) showed different quality attributes from the CPO obtained by traditional methods of extraction (Mba et al., 2015).

Traditionally, the oil is isolated by several methods including boiling the fruit, pounding and pressing or suspending the sludge in hot water. Modern methods of extraction of palm oil from the fruits are more efficient and include the following steps: cooking, pressing, centrifugation and filtration under vacuum. Palm fruits may be subjected to strong enzymatic hydrolysis and microbial degradation during harvest and handling prior to extraction of CPO. This scenario is very common with traditional practices in Nigeria, and has been responsible for high FFA in HFFA CPO. The estate palm oil (LFFA CPO) which is produced by modern milling technology has low FFA values and impurities because the fruits are harvested and immediately processed and extracted in the factory without long periods of storage (Mba et al., 2015).

The quality of palm oil can be poor unless the source of palm fruits are handled carefully and promptly to minimize the impact of agents of spoilage such as air, water, enzymes and micro-organisms (Sonntag, 1979). It has been reported that poor quality palm oil can be processed and cleaned by washing the oil with hot water, followed by filtration and centrifugation. This process eliminates impurities, high FFA and moisture content and improves the stability of the final product (Igile et al., 2013). As was earlier stated, the quality of palm oil determines its market acceptability and price, and this correlates directly with the free fatty acid (% FFA) concentration and the total fatty matter (% TFM) content of the commodity. Therefore, businesses using palm oil for production are very particular about the moisture content, FFA, impurity and TFM contents of CPO. Total contaminants including moisture, unsaponifiable matter and other impurities add to the free fatty acid content of any oil to significantly reduce the available % TFM content of the oil (Saad et al., 2007). It has been reported that the degradation action of lipase increases FFA levels in CPO, which is considered a very important quality parameter because FFA concentration is one of the most important characteristic quality index for the storage time, marketing, production, and price of palm oil (Saad et al., 2007; Baterman et al., 2010).

Palm oil contains several saturated and unsaturated fatty acids. Irrespective of the grade of CPO, the fatty acids composition of crude palm oil have been reported to include capric, caproic, caprylic, lauric, myristic, palmitic, stearic, oleic, linoleic and linolenic acids. Palmitic acid has been reported to be the dominant fatty acid in CPO (40-45%) and to be the most widely distributed saturated fatty acid in vegetable oils and animal fats to the extent of at least 5%. It was reported that the oleic acid content in CPO is also significant (36-40%) (Japir et al., 2017).

The lower members of saturated fatty acids are liquids at room temperature, whereas those containing 10 or more carbon atoms are solids having progressively higher melting points (titre, °C) with increasing length of carbon chain. It has been reported that the even progression of melting points and the length of the carbon chain gives a smooth curve only if the even-membered homologues are considered. When all the fatty acids are included, lower than that of the even-chain acid immediately before it (Sonntag, 1979).

Palm oil is rich in carotenoids including β-carotene which is a precursor of Vitamin A. Palm oil has been used for Vitamin A deficiency interventions and has therefore been used for preventing Vitamin A deficiency, cancer,

brain disease, and aging. It is also used to treat malaria, high blood pressure, high cholesterol level, and dementia and cyanide poisoning. Palm oil is used for weight loss and for increasing the body's metabolism (Mancini et al., 2015). As food, palm oil is used as cooking oil and for frying, and it is also an ingredient in many processed foods. Industrially, palm oil is used for the manufacture of cosmetics, soaps, detergents, toothpaste, waxes, lubricants and ink. However, excessive intake of palm oil has been reported to predispose hypercholesterolemia, hyperlipidemia and diabetes with attendant high concentrations of low density lipoprotein cholesterol (LDH-Cholesterol) (Mancini et al., 2015).

The aim of the present study was to assess the quality of LFFA CPO and HFFA CPO samples from different locations in Southern Nigeria produced through traditional and modern extraction methods, and to determine their respective TFM content aimed at assisting statutory regulatory activities.

MATERIALS AND METHODS

Collection of plant

Fresh LFFA CPO samples aged 5 to 10 days from estate palm oil mills were purchased from four locations in Nigeria including, Port Harcourt (SPORP), Calabar (SPOCAL), Benin (SPOOK), and Ibadan (SPOIB) and stored in 2-L brown bottles under refrigeration until required. Also samples of HFFA CPO were purchased from the same locations in an open retail market after ensuring that they were traditionally processed. The HFFA CPO samples were also 5 to 10 days old and were labelled Port Harcourt (TPORP), Calabar (TPOCAL), Benin (TPOOK), and Ibadan (TPOIB). All samples were properly labelled and identified, and stored in brown glass bottles under refrigeration (16±2.0°C) to minimize further deterioration and production of FFA.

Sample preparation for physico-chemical analysis

Two hundred grams of each sample of LFFA and HFFA CPO was measured and labelled according to the labelling earlier described and stored under refrigeration (16±2.0°C) for physico-chemical analysis including, moisture content, free fatty acid, impurity, unsaponifiable matter, saponification value, peroxide value, iodine value, hydroxyl value, acid value, total fatty matter, titre or melting point and the lovibond colour of oil samples (using 51/4" cell at 55°C). All parameters were analysed using to standard analytical procedures.

Determination of moisture content of LFFA and HFFA CPO sample

The moisture content of the LFFA and HFFA CPO samples was determined using the Karl Fischer method (AOCS, Tb 2-64; Emery, 1983). The fundamental principle in this method is to determine the actual water content of the oil by titration of the oil sample with Karl Fischer reagent which reacts quantitatively with water.

Determination of impurity/dirt in LFFA and HFFA CPO samples

Five (5) grams of oil sample was heated to a temperature of 105°C in a Gallenkamp oven. The oil was filtered through Whatmann No. 4 Filter paper, previously dried and weighed to a constant weight. The filter paper was then dried after oil filtration to constant weight. The impurity content was determined from the equation:

$$\text{Impurity (\%)} = (W_2 - W_3 / W_1) \times 100$$

where W_1 = weight of oil sample, W_2 = initial weight of filter paper, and W_3 =final weight of filter paper.

Determination of colour of raw LFFA and HFFA CPO samples

The colour of LFFA and HFFA raw oil samples was determined using the Lovibond method described by the British Standard Institute method BS 684 using the Lovibond Tintometer Model E AF900 (Tintometer Ltd, Salisbury, UK). In this method, 100 ml of raw oil was heated to 70°C in a Gallenkamp oven at 105°C. The oil was allowed to cool to 55°C, and measured at 55°C using $5\frac{1}{4}$ inch cell in a lighted Lovibond Tintometer against known standard glass colours. The colour of oil was expressed as (RYBN), meaning Red (R), Yellow (Y), Blue (B) and Neutral (N) for both LFFA and HFFA CPO samples.

Determination of free fatty acid (% FFA) of LFFA and HFFA CPO samples

Free fatty acid (FFA) of LFFA and HFFA CPO samples was determined using the AOCS method Ca 5a-40 (1989). In this method 5 g of oil was placed in a 250 ml Erlenmeyer flask. Then 50 ml pre-neutralized isopropyl alcohol (IPA) was added and mixed thoroughly and 3 drops of phenolphthalein was added and mixed. The mixture was placed on a hot plate with a magnetic stirrer and heated to 40°C, and then titrated with 0.1N NaOH to the phenolphthalein end point of a stable pink solution.

$$\%\text{FFA} = \frac{\text{Vol. of titrant} \times (N) \text{ of titrant} \times 25.6}{\text{Weight of oil sample (g)}} \times \frac{\text{mg NaOH}}{\text{Sample (g)}}$$

where, 25.6 is the FFA conversion factor for palmitic acid.

Determination of acid value (AV) of LFFA and HFFA CPO samples

Acid values (AV) of LFFA and HFFA CPO samples were determined by multiplying individual % FFA with 2.19 which is the conversion factor for palmitic acid.

$$\text{Acid Value (AV)} = \% \text{ FFA} \times 2.19;$$

where 2.19 is the conversion factor for palmitic acid.

Determination of iodine value (IV) of LFFA and HFFA CPO samples

Iodine value (IV) of the LFFA and HFFA CPO samples was determined using the Wij's method (BS 684; Section 2.13, 1976) as described by Abdullah et al. (2013). Briefly, 4 g of oil sample was placed in a 500 ml Erlenmeyer flask and 15 ml of cyclohexane was added and shaken to dissolve the oil. Then 25 mL of Wij's reagent was added and mixed and place in the dark with periodic mixing for 1 h, and 150 mL of distilled water was added to the mixture and shaken to mix. Finally, 20 mL of 10% KI solution was added to the mixture and titrated against 0.1N Na2S2O3 to obtain a stable

yellow colour to which 1 mL of 1% starch solution was added. The titration continued to obtain a stable blue colouration. The procedure was repeated using a blank solution of distil water.

Iodine value (IV) was calculated according to the equation:

$$IV = \frac{(Vb\text{-}Vt) \times (N) \text{ of titrant} \times (12.69)}{\text{Weight of sample (g)}} \times \frac{I2\ (g)}{\text{Sample (100 g)}}$$

where Vb = mL of blank, Vt = mL of titrant, and 12.69 is the equivalent conversion factor from thiosulphate to I2.

Determination of hydroxyl value (HV) of LFFA and HFFA CPO samples

The method described by Fernandes et al. (2014) was adopted. Briefly, 5 g of oil sample was placed in a 250 mL conical flask and 5 mL of a reagent mixture of acetic anhydride/pyridine, 1:4 v/v was added and mixed. The mixture was refluxed at 100°C for 1 h with constant stirring using a magnetic bar, and then cooled to ambient room temperature (27±0.50°C) and 10 ml of deionized water was added to complete the hydrolysis of excess acetic anhydride in the mixture. The mixture was further refluxed for about 10 min, then cooled to room temperature, followed by the addition of 25 mL neutralized ethanol and 3 drops of phenolphthalein indicator. This was titrated against 0.5N methanolic KOH to obtain a light pinkish colouration. Hydroxyl value was obtained according to the equation:

$$IV = \frac{(V_b\text{-}Vt) \times (N) \text{ of titrant}) \times (12.69)}{\text{Weight of sample (g)}} \times \frac{I_2\ (g)}{\text{Sample (100g)}}$$

where Vb = mL of blank, Vt = mL of titrant; AV= Acid Value, and 56.1 is a factor for the conversion of hydroxyl value.

Determination of saponification value (SV) of LFFA and HFFA CPO samples

The SV of the oil samples was determined using the BS 684 2.6 (1977) method described by Abdullah et al. (2013). In this method 3 g of oil sample was placed in a 250 mL conical flask, and 25 mL 0.5 N ethanolic KOH was added and mixed by shaking and refluxed for 1 h by boiling over a hot plate and stirring with a magnetic bar. The mixture was allowed to cool slightly and 3 drops of phenolphthalein was added and mixed and then titrated against 0.5 N HCL to a pink colouration end point. Saponification value (SV) was calculated using the equation:

$$SV = \frac{(V_b\text{-}Vt) \times (N) \text{ of titrant}) \times (56.10)}{\text{Weight of sample (g)}} \times \frac{mgKOH}{g\ sample}$$

where Vb = mL of blank and Vt = mL of titrant.

Determination of peroxide values (PV) of LFFA and HFFA CPO samples

Peroxide values (PV) of the LFFA and HHFFA CPO samples were determined using the improved AOAC mFOX method described by Burat and Bozkurt (1996). Briefly, 0.2 g of oil sample was weighed into 100 ml conical flask into which a mixture of 9.8 m < chloroform/methanol (7:3) was added and mixed briefly. The mixture was transferred into a screw-capped vial and 100 µL of 10 mM xylenol orange was added and mixed by shaking for about 20s. Then 50 µL of 36 mM iron (111) chloride solution was added and mixed. The solution obtained was allowed to stand for 5 min at room temperature (27±0.50°C). The absorbance of each sample was read at 560 nm wavelength using a UV/VIS spectrophotometer (Model; Schimadzu UV-1700). The PV was extrapolated from a previously prepared standard curve. The standard calibration curve was prepared from a solution of iron (111) chloride (10 ug/ml) in 9.8 mL chloroform/methanol (7:3) mixture. A serial dilutions ranging from 5 to 30 ug/L was prepared and plotted against the absorbance to produce a linear curve from which the respective PV of the oil samples was extrapolated.

Determination of ester value (EV) of LFFA and HFFA CPO samples

The ester values (EV) of the LFFA and HFFA CPO were calculated for each oil sample according to the equation,

EV = SV-AV

where SV is the saponification value and AV is the acid value of the oil sample, respectively.

Determination of %TFM of LFFA and HFFA CPO samples

Total fatty matter (as %TFM) was calculated for each oil sample according to the equation:

%TFM = 100% – (FFA + Moisture + Impurity + Unsaponifiable Matter)% × (95/100)

where 95% is the absolute TFM of clean Oils and conversion factor for % TFM of oil.

Composite oil samples preparation for GC-MS analysis of fatty acids

Four grams of oil was measured from each LFFA CPO sample and mixed to produce 20 g of composite LFFA CPO sample for the derivatization of FAMEs for GC-MS analysis. The same procedure was replicated with HFFA CPO samples for GC-MS analysis.

Fatty acids methyl esters (FAMEs) derivatization of the oil samples for GC-MS analysis

Twenty five (25) ng of oil sample was weighed into 10 mL micro-reaction vessel and 2 mL BCl3-MeOH 12% w/w was added. This was followed by the addition of 1 mL 2, 2-dimethoxyproprane. The mixture was mixed thoroughly and then heated for 5 min at 60°C. It was then cooled to below 30°C and 1 mL distilled water and 1 mL n-hexane was added and mixed thoroughly again, then allowed to stand for 10 min. The upper (organic) layer was pipetted into a sterile and clean vial and covered with a stopper and stored for GC-MS analysis. This procedure was replicated in all the LFFA and HFFA CPO samples.

GC-MS analysis of FAMEs for fatty acid composition of SPO and TPO samples

The method reported by Dodds et al. (2005) and modified by Igile et al. (2020) was used to carry out the GC-MS analysis. The sample of FAME prepared from each oil sample, was injected manually through the injector pot of the Agilent 6890 GC coupled

Table 1. Physico-chemical quality characteristics of Nigerian crude palm oil samples (LFFA and HFFA).

Parameter	SPOCAL	SPOIB	SPORP	SPOOK	TPOCAL	TPOPH	TPOBN	TPOIBA
Moisture (%)	0.53±0.04^{a}	0.60±0.05^{a}	0.55±0.02^{a}	0.45±0.02^{a}	1.02±0.25^{c}	1.63±0.07^{b}	1.47±0.40^{b}	1.65±0.05^{c}
FFA (% Palmitic acid)	2.44±0.30^{a}	2.81±0.21^{a}	2.95±0.08^{a}	2.65±0.20^{a}	9.25±0.70^{bc}	12.54±0.45^{b}	10.52±0.85^{b}	12.76±1.20^{c}
Impurity (%)	0.05±0.01^{a}	0.11±0.03^{a}	0.04±0.02^{ac}	0.02±0.01^{ac}	0.24±0.05^{c}	0.44±0.03^{b}	0.39±0.03^{b}	0.35±0.02^{b}
Unsap Matter (%)	0.27±0.04	0.30±0.05	0.29±0.07	0.24±0.03	0.41±0.11	0.59±0.09	0.47±0.05	0.45±0.06
Sap Value (mgKOH/g oil	199.95±0.8^{a}	199.87±0.5^{a}	199.92±0.6^{a}	199.88±0.9^{a}	197.57±0.8^{a}	197.52±0.7^{a}	196.55±0.5^{a}	196.62±0.4^{a}
Peroxide Value (meq/kg)	5.25±0.45^{a}	5.44±0.30^{a}	5.27±0.25^{a}	4.79±0.11^{a}	1.77±0.03^{c}	1.45±0.02^{b}	1.56±0.05^{b}	1.90±0.06^{c}
Iodine Value (Wijs)	56.25±0.85^{b}	57.25±0.92^{b}	57.83±0.75^{b}	56.55±0.64^{b}	52.11±1.20^{a}	53.32±1.95^{c}	52.65±1.13^{a}	53.42±1.25^{a}
Acid Value (mgKOH/g)	6.72±0.12^{c}	7.87±0.35^{a}	7.92±0.45^{a}	7.21±0.37^{a}	9.77 ± 0.17^{b}	9.32 ± 0.25^{b}	8.57 ± 0.45^{b}	9.68 ± 0.29^{b}
Hydroxy Value	17.22±0.55^{a}	16.41±0.39^{a}	16.55±0.15^{a}	16.92±0.42^{a}	24.28±0.25^{b}	27.45±0.34^{c}	25.62±0.46^{c}	27.35±0.09^{b}
Ester Value (mgKOH/g)	193.23±1.1^{a}	190.00±0.9^{a}	192.00±0.92^{a}	192.67±1.4^{a}	187.80±1.8^{a}	188.20±1.2^{a}	187.98±0.7^{a}	186.94±1.5^{a}
Titre (^{o}C)	45.05±0.41^{a}	45.00±0.05^{a}	44.95±0.04^{a}	44.90±0.05^{a}	43.75±0.25^{b}	44.11±0.45^{b}	44.05±0.07^{b}	43.85±0.15^{b}
TFM (%)	92.45±0.75^{a}	91.95±0.82^{a}	91.94±0.40^{a}	92.39±0.37^{a}	85.16±1.05^{b}	81.07±0.31^{bc}	83.32±0.92^{bc}	81.06±0.64^{c}
Lovibond Colour (5^{1}/4" Cell)	18R12Y7B^{a}	20R13Y7B^{a}	19R13Y6B^{a}	18R12Y5B^{a}	23R15Y9B	23R14Y12B	24R15Y12B	25R15Y10B

Values are expressed as Mean ± SD from triplicate samples test results (n=3). Different superscripts letters within the same row are significantly ($p<0.05$) different.

with a 5973i mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) which was connected to a ChemStation Integrator to interpret data. The GC was equipped with a HP-5MS capillary column (30 m × 250 µm i.d. × 0.25 µm, Agilent Technologies). Helium was used as the carrier gas with a constant flow rate of 1 mL/min to the column. The initial oven temperature was set at 40°C, holding for 2 min, then raised to 150°C at 5°C/min; and finally raised to 280°C at 15°Cmin^{-1}, holding for 2 min. The injection pot was maintained at splitless mode. The mass detector was operated at 150°C in electron impact (EI) mode at 70 eV. The ion source temperature was at 230°C and the transfer line temperature was maintained at 250°C. The chromatograms were recorded by monitoring the total ion currents in the 15 to 450 mass range. MS was detected with 2 min solvent delay. Analysis of each sample at each condition was repeated twice to ensure consistency. C6-C24 *n*-alkanes were run under the same chromatographic conditions in order to calculate the retention indices (RI) of detected compounds. Identification of fatty acids and other volatile constituents were based on retention indices relative to n-alkanes (C6-C24), and computer matching with the WILLEY 275.L library, and those contain in the NIST08 database; and confirmed by comparison of the retention times reported in literature (Igile et al., 2020).

Statistical analysis

Physico-chemical determinations were carried out in triplicates while GC-MS determinations were done in duplicates and results were expressed as Mean ± SEM, for n=2 or n=3, and subjected to analysis of variance (ANOVA) to check for statistical significance ($p<0.05$).

RESULTS AND DISCUSSION

Physico-chemical evaluation of LFFA and HFFA CPO

The comparative results of the physico-chemical evaluation of LFFA and HFFA CPO samples are presented in Table 1. The physico-chemical properties of CPO including, appearance, odour and taste determine its quality and consumer acceptability (Igile et al., 2013). The length of time between fruits harvest and processing, type of processing method employed, storage and handling conditions, may significantly affect the moisture content, impurities, unsaponifiable matter and free fatty acid content of both LFFA and HFFA CPO. These parameters contribute significantly to the overall quality of palm oil in the market place (Fakou et al., 2009; Igile et al., 2013; Japir et al., 2017). Modern palm oil (PO) mills and improved technologies have successfully been applied to produce high grade palm oil of low FFA, impurity and moisture values, and this type of oil has been graded as LFFA CPO (Table 1). The FFA values of the LFFA CPO samples were significantly ($p<0.05$) lower and stable than values for HFFA CPO (Figure 1). The range of FFA% values for LFFA CPO was 2.44±0.30 -2.95±0.08%, whereas mean FFA values for HFFA CPO was in the range 9.25±0.70 - 12.76±1.20%. The moisture and FFA values obtained for HFFA CPO in the present study were consistent with the values reported by Ohimain et al. (2012). However,

Figure 1. Map of Nigeria showing cities sampling and study locations. Sampling areas are circled in the map. SPORP = SPO Rison Palm, SPOCAL = SPO Calaro Palm, TPOCAL = TPO Calabar Market, TPOBN = TPO Benin Market, SPOOK = SPO Okomu, SPOIBA = SPO Ibadan Palm, TPOPH = TPO Port Harcourt Market, TPOIBA = TPO Ibadan Market.

Ohimain et al. (2012) reported very high impurity levels of between 5.48 and 12.52% when compared with the values (0.24-0.44%) obtained for HFFA CPO in this study. We compared the values of some physico-chemical parameters obtained in this study with those obtained by Adebayo-Oyetoro et al. (2019). Their study reported unusually high values of FFA (14.70- 21.45%) and unusually low moisture values (0.38-2.41%) which did not correlate with one another. Such low moisture values cannot give such high FFA values. They also reported IV values of 84.94-179.71 which was inconsistent with the entire physico-chemical results in that study. The IV values did not correlate with the FFA and moisture values reported and known structural and ageing chemistry parameters of CPO.

Modern mills from Calabar gave the lowest FFA values for LFFA (2.44±0.30%) and HFFA CPO (9.25±0.70%), respectively. Port Harcourt LFFA CPO (SPORP) sample gave the highest mean FFA% value (2.95±0.08%), while Ibadan HFFA CPO (TPOIB) gave the highest mean FFA value (12.76±1.20%). Traditional milling and processing methods among the natives in Nigeria are known to be crude and thus produce HFFA CPO grades with significantly ($p<0.05$) high FFA values (>5.00%) (Figure 1). The factors affecting the traditional methods of processing include delays in processing harvested fruits and the use of crude processing and extraction methods such as boiling of fruits, extraction with hot water under unhygienic conditions, resulting in enzymes and microbial-aided fruits spoilage, bad odour, high moisture, poor colour, high impurity and unsaponifiable matter, as well as high FFA values of CPO (Table 1).

HFFA CPO samples from traditional production always present with significantly ($p<0.05$) low available TFM (81.06±0.64-85.16±1.05%), when compared with LFFA CPO with significantly high available TFM values (91.94±0.40 - 92.45±0.75%) (Table 1) processed from modern mills with improved technologies. Therefore, the concentration of FFA, moisture, impurity and unsaponifiable matter significantly contributes to available %TFM of both LFFA and HFFA CPO (Figure 2), and this affects the industries utilizing the oil as raw material for production (Igile et al., 2013). Poor quality oil results in increased cost of processing. High values of the quality parameters of CPO give rise to lower available %TFM in HFFA oil (Figure 2). Thus, production of palm olein, butter and salad dressings in the food industry will incur losses because of low available TFM resulting from high concentrations of key physico-chemical parameters in HFFA CPO. Also manufacturers of soap, detergents and cosmetics will incur losses because of the availability of low TFM in HFFA oil. Consequently, organizations around the world utilizing CPO for production insist on buying LFFA grade CPO. LFFA CPO contains significantly ($p<0.05$) low concentrations of physico-chemical parameters including FFA, moisture, impurity and unsaponifiable matter (Figure 1) resulting in high available TFM content (Figure 2). It has been reported that high

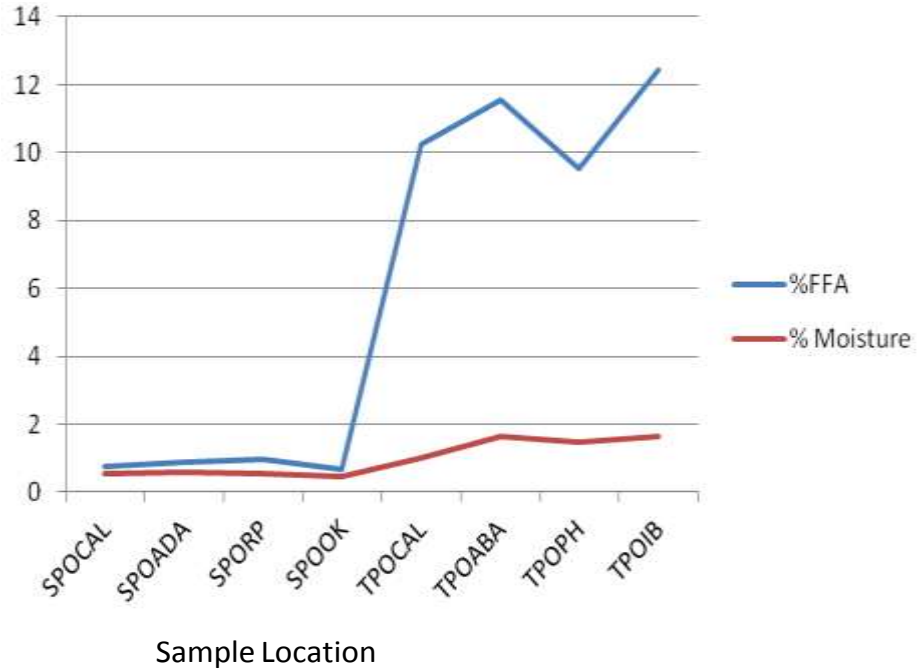

Figure 2. Variation of %FFA and moisture values with oil type and sample location.

hydrocarbons, tocopherols, carotenoids, plant sterols, gums and tocotrienols constitute what is referred to as unsaponifiable matter. These are substances which are dissolved in the CPO but cannot be saponified by caustic alkali (Prasanth and Gopala, 2014).

The saponification value (SV) of oil is a measure of the average molecular weight of oil or all fatty acids in the oil. It can be defined as the number of milligrams of KOH required to saponify 1 g of oil or fat under the conditions specified (Salimon et al., 2012). The acid value of oil just like the FFA% is an indication of the ageing state or degradation of the oil (Saad et al., 2007). It is therefore the number of mg of KOH required to neutralize the free fatty acid in 1 g of sample. Thus SV (or, precisely, the AV of oil or fatty acids) and the fatty acid ratio are both measures of the average equivalent mass, and the average chain length of the mixture of fatty acids in the oil.

The SV, AV and EV are related by the equation:

EV = SV–AV;

Where EV is the ester value which is a measure of the glyceride present in the oil sample. Also SV and AV are important parameters in the estimation of the glycerol content of a fat or oil. The average degree of unsaturation of a fat or mixture of fatty acids is measured by the iodine number or iodine value (IV) and is expressed in terms of the number of centigrams of iodine absorbed per g of sample in a Wijs reaction (British Standards: BS 684 Section 2.13; 1976).

Variation of TFM in LFFA and HFFA CPO samples

The %TFM was expectedly found to be higher in LFFA CPO samples and significantly lower ($p<0.05$) in the HFFA CPO samples studied (Figure 3). Calabar SPOCAL gave the highest mean TFM value of 92.45±0.75% while Port Harcourt SPOPH gave the lowest mean TFM value of 91.94±0.40%. With respect to the %TFM values of HFFA CPO samples, Calabar (TPOCAL) gave the highest mean %TFM value of 85.16±0.05 while Ibadan (TPOIBA) gave significantly lower mean %TFM value of 81.06±0.64% ((Figure 3).

Total fatty matter was relatively stable in the LFFA CPO samples and was found within the range (91.94±0.40 92.45±0.75%) when compared with the large variation of mean %TFM in HFFA CPO samples (81.06±0.64 - 85.16±0.05%) (Figure 2). It was observed that the differences in processing methods and impurities contents in both grades of CPO accounted for the difference in %TFM found between the two grades of CPO studied. Differences in cultural practices and processing methods between eastern and western Nigeria may also have contributed to the variation in the TFM of the HFFA CPO samples studied.

Fatty acid composition of LFFA and HFFA CPO samples

The fatty acid composition of both grades of CPO samples was determined by GC-MS method. The

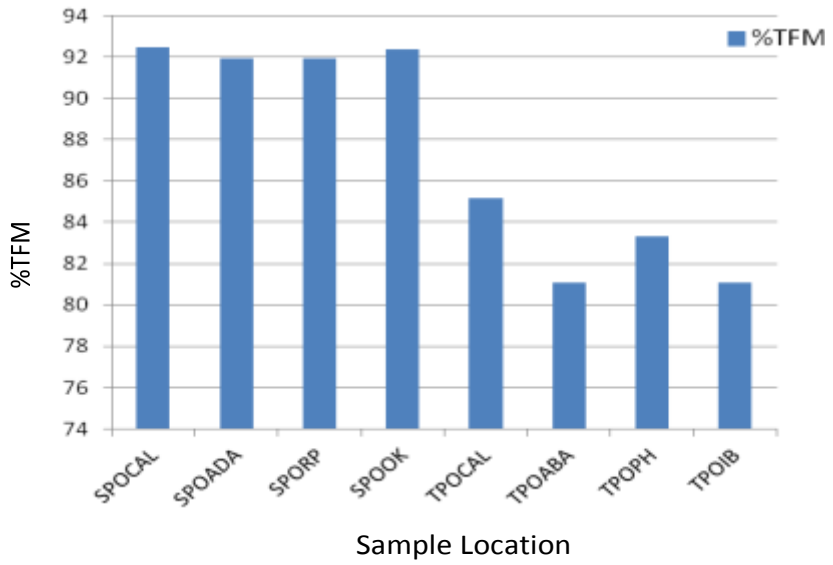

Figure 3. Variation of %TFM values with oil type and sample location.

Table 2. Fatty acid distribution of composite sample of LFFA CPO from *Elaeis guinensis* (Jacq.) purchased from retail market stores in Nigeria.

Peak no.	RT (min)	Name of compound	Molecular formular	Abundance (%)
1	1.441	Caprylic acid	$C_8H_{16}O_2$	0.119±0.05
2	2.652	Capric acid	$C_{10}H_{20}O_2$	0.355±0.03
3	3.112	Lauric acid	$C_{12}H_{24}O_2$	0.591±1.02
4	3.524	Myristic acid	$C_{14}H_{28}O_2$	0.792±0.45
5	4.710	Palmitic acid	$C_{16}H_{32}O_2$	45.641±1.77
6	10.625	Stearic acid	$C_{18}H_{36}O_2$	3.452±0.08
7	11.911	Palmitoleic acid	$C_{16}H_{30}O_2$	0.491±0.55
8	14.260	Oleic acid	$C_{18}H_{34}O_2$	38.005±1.06
9	14.871	Linoleic acid	$C_{18}H_{32}O_2$	10.440±0.25
10	15.035	Linolenic acid	$C_{18}H_{30}O_2$	0.511±0.04
			Total	100.465±3.11

Values are expressed as mean ± SEM, n = 2.

fatty acid composition of fats and oils is generally determined by conversion of the fat or oil by methanolysis to mixed fatty acid methyl esters (FAMEs) followed by analysis with GC-MS. The summary of the GC-MS results for the LFFA CPO composite sample is presented in Table 2, while that of HFFA CPO sample is presented in Table 3.

The GC-MS analysis gave the fatty acid composition for LFFA CPO as follows: caprylic acid (0.119±0.05%), capric acid (0.355±0.03%), lauric acid (0.591±1.02%), myristic acid (1.792±0.45%), palmitic acid (45.641±1.77%), stearic acid (3.452±0.08%), palmitoleic acid (0.491±0.55%), oleic acid (39.005±1.06%), linoleic acid (10.440±0.25%), and linolenic acid (0.511±0.04%). The GC-MS results for HFFA CPO composite sample were, caprylic acid (0.167±0.07%), capric acid (0.438±0.05%), lauric acid (1.114±1.25%), myristic acid (1.725±0.65%), palmitic acid (44.670±0.85%), stearic acid(3.050±0.55%), palmitoleic acid (0.450±0.72%), oleic acid (37.370±1.06%), linoleic acid (10.420±0.40%), and linolenic acid (0.250±0.05%).

The fatty acid composition and distribution in the palm oil samples studied were consistent with results earlier reported (Derawi et al., 2014; Japir et al., 2017). The saturated fatty acids (SFAs) found in this study were common to both grades of CPO and included capryllic, capric, lauric, myristic, palmitic and stearic acid. These fatty acids constituted the TSFAs

Table 3. Fatty acid Distribution of Composite sample of HFFA CPO from Elaeis guinensis (Jacq.) purchased from Retail Market Stalls in Nigeria.

Peak no.	RT (Min)	Name of compound	Molecular formular	Abundance (%)
1	1.279	Caprylic acid	$C_8H_{16}O_2$	0.167±0.07
2	2.124	Capric acid	$C_{10}H_{20}O_2$	0.438±0.05
3	2.677	Lauric acid	$C_{12}H_{24}O_2$	1.114±1.25
4	3.908	Myristic acid	$C_{14}H_{28}O_2$	1.725±0.65
5	4.522	Palmitic acid	$C_{16}H_{32}O_2$	44.670±0.85
6	10.390	Stearic acid	$C_{18}H_{36}O_2$	3.050±0.55
7	11.490	Palmitoleic acid	$C_{16}H_{30}O_2$	0.450±0.72
8	13.591	Oleic acid	$C_{18}H_{34}O_2$	37.370±0.92
9	13.972	Linoleic acid	$C_{18}H_{32}O_2$	10.420±0.40
10	14.515	Linolenic acid	$C_{18}H_{30}O_2$	0.250±0.05
			Total	100.955±3.782

Values are expressed as mean ± SEM, n = 2.

Table 4: Composition of Saturated, Monounsaturated and Polyunsaturated Fatty Acids in LFFA and HFFA CPO Samples.

Fatty Acid Type	Type of Oil	
	SPO	TPO
Total SFAs (%)	49.950±1.72	51.164±2.05
Total MUFAs (%)	38.496±1.43	37.820±0.75
Total PUFAs (%)	10.951±0.45	10.670±0.52
Summary		
Total SFAs (%)	49.950±1.72	51.164±2.05
Total TUFAs (%)	49.447±1.09	48.490±1.25
TSFAs **:** TUFAs	1:1	1:1

Values are expressed as mean ± SEM, n = 2
SFAs = Saturated fatty acids; MFUs = Monounsaturated fatty acids; PUFAs = Polyunsaturated fatty acids; TSFAs = Total saturated fatty acids; TUFAs = Total unsaturated fatty acids

of 49.950±1.72% in LFFA CPO and 51.164±2.05% in HFFA CPO, respectively (Table 4). In both grades of CPO samples, the major saturated fatty acid was palmitic acid (C16:0).

Oleic acid (C18:1) was the major monounsaturated fatty acid (MUFA) found in RPO in this study, accounting for 38.496±1.43% in LFFA CPO and 37.820±0.75% in HFFA CPO. Linoleic and linolenic acids were common to both grades of oil samples as polyunsaturated fatty acids (PUFAs). The total PUFAs in LFFA CPO was 10.951±0.45% and 10.670±0.52% in HFFA CPO, and showed no significant difference in concentration between the two grades of CPO (Table 4). The total unsaturated fatty acids (TUFAs) in LFFA CPO were 49.447±1.09 and 48.490±1.25% for HFFA CPO. Linoleic acid (C18:2) and linolenic acid (C18:2) which are essential FAs accounted for over 10% of the fatty acid contents of both grades of CPO. The presence of linoleic acid at about 10% has favourable nutritional implications and beneficial physiological effects in humans.

Linoleic acid prevents coronary heart disease and cancer and provides lipids necessary for cell membrane repair and cellular respiration (Oomah et al., 2000). The ratio of total saturation to total unsaturation in both LFFA CPO and HFFA CPO was found to be 1:1 (Table 4). The dietary and nutritional significance of this balance in ratio between total saturation and total unsaturation in CPO is not very clear and has not been investigated or reported in literature, and this may be a subject of further investigation.

Conclusion

It was concluded that CPO is rich in SFAs, MUFAs and

PUFAs and the ratio of TSFAs to TUFAs for both LFFA and HFFA CPO is 1:1, and this may have dietary and nutritional significance. The available TFM in LFFA CPO was significantly higher than in HFFA CPO. The quality of both grades of oils did not affect their fatty acid composition and distribution; neither did it affect the SV, EV and IV but affected AV (an indicator of the FFA content of vegetable oils).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Review

Introducing cotton farming by the use of transgenic cotton for phytoremediation of industrial wastes polluted soils in Southern Nigeria

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Industrialization in the Niger Delta region of southern Nigeria has resulted in excessive discharge of industrial wastes, especially oil spillage. Farming in this region has declined and cotton farming has not survived there due to this menace. Phytoremediation, which is an emerging green technology has been adopted for the revitalization of farming in polluted sites and has been integrated towards solving this challenge. Many indigenous plants of high phytoremediation strength within and outside Nigeria have been reported. The use of genetic engineering approaches for developing transgenic plants with higher phytoremediation potential have also been successful in certain plants. This review, therefore, focused on phytoremediation, its impact, success, potential plants, plant's phytoremediation mechanisms, and the technological advancement need through cotton genetic engineering. Cotton is the foremost commercially important fiber crop and its fiber is the backbone of the textile industry. It has significant impact in the economy but its phytoremediation strength is naturally poor; hence, prompting attention to the genetic modification of cotton for phytoremediation purposes and basing the future phytoremediation on the use of transgenic economic plants, especially cotton, are of significant importance.

Key words: Pollution, farming, cotton, phytoremediation, genetic engineering.

INTRODUCTION

Cotton is an important rare economic success story in sub Saharan Africa, a major source of foreign exchange earning in more than 15 countries of the continent and a crucial source of income for millions of rural people (Nnaemeka and Sun, 2021). It is considered as the foremost commercially important fiber crop and is deemed as the backbone of the textile industry (Chakravarthy et al., 2014). Therefore, issues surrounding cotton have become of serious concern to the world. In the past, there was decline in cotton farming as a result of pest infestation and other related factors. The revitalization of cotton for its fiber was a huge scientific exercise survived by the discovery of *Bacillus thuringiensis Bt*. by Shigetane in 1901. Berliner in 1915

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reported the existence of a crystal within the *Bt.* while Hannay et al. (1956) found that the main insecticidal activity against Lepidoptera (moth) insect was due to the parasporal crystal. In 1995, it became feasible to move the gene that encodes the toxic crystals into some plants in which cotton was among the genetically engineered plants by Monsanto (USA). (Monsanto 2002b). Cotton therefore, has been successfully genetically manipulated to resist insect attack. However, other issues regarding the quality of the cotton fiber have been approached because the price received for the cotton is dependent on the quality of each cotton bale, long, uniform, strong, fine mature cotton fiber provides better returns to growers and open more market opportunities for premium fabrics (Nnaemeka and Sun, 2021). Despite the already solved pest infestation challenge, in terms of fiber length and micronaire, Nigerian cotton was found at (Grade 5) compared to Australian cotton of (Grades 6 and 7), respectively in the international market. The limitation of Nigeria cotton fiber inspired interest in working on cotton plant (Upland cotton) at Plant Genomics Lab of Zhejiang Sci-Tech University (ZSTU) and we focused on combining traits of cottons towards genetically improving the fiber quality of the upland cotton (*Gossypium hirsutum* L.) which is the dominant species in Nigeria. Successes recorded from the research are expected to enhance the quality of the cotton fiber and improve effort towards integration of the genetic approaches used in cotton farming. However, following observations from excursion to some of the farming sites in Nigeria, we realized that the efforts made towards improvement of the cotton fiber quality have met another serious challenge (land pollution) that hinders cotton farming.

Generally, the current challenge farming is facing in the Southern Nigeria is due to spillage. High concentration of metal (loids) and organic pollutants in the contaminants are released into the soil, presenting a global threat to the surrounding environment and human health (Gordana et al., 2018). These contaminants are discharged from various processes which also include the printing and dying processes in the textile industry that contain high concentration of carcinogenic halides (Gao et al., 2019). Studies have disclosed the harmful effects of such chemicals to aquatic and terrestrial lives and also the climate. Led by Prof. Sun Yuqiang at ZSTU Plant Genomics lab, we used molecular biology approach to develop naturally colored cotton fibers yarn without or very less dying directly into cloth, matching the increasing great demand for green products, environmental protection and human health in modern society (Gao et al., 2019). Currently, only brown and green colored cotton is available in the actual production which seriously restricts the development of colored cotton industry and paves the way for continuous dyeing process that are constituting environmental havoc (Gao et al., 2019). Also in the nearby refinery industries, limited efforts have been made to curb discharge of similar obnoxious substances. Also, poor waste disposal from domestic activities resulted to the persistent decline in agricultural activities, especially in cotton farming which is of great economic importance. Although, heavy metal (loids) occurs naturally in the environment from pedogenetic of weathering of parent materials and also through anthropogenic sources, the most significant natural sources are weathering of minerals, erosion and volcanic activity, while the anthropogenic sources depend upon human activities such as spillage, mining, smelting, electroplating, use of pesticides and phosphate fertilizer discharge as well as biosolids (Ruchita et al., 2015). Government of Nigeria have so far initiated a clean-up program to clean the affected farm land but this effort have yielded minimal result and the decline in farming as a result of contamination from these activities is still increasing. Because Nigeria is the most populous and economy rich country in Africa as well as a major contributor to Africa economy, and cotton has significant impact on its economy growth (Nnaemeka and Sun, 2021), issues surrounding its economy, agriculture and environment are usually of serious concern. The quest for contribution to solving these challenges inspired this review. Here, an outlook of the depreciating farming activities in Southern Nigeria due to spillage is presented and the role of phytoremediation in revitalization of the farming situation is discussed. The paper highlights challenges facing agriculture and precisely cotton farming in Nigeria and even Africa and demonstrates how phytoremediation and genetic engineering approaches are the key to sustaining farming, maintaining healthy ecosystem and odor free environment. It also provides national and regional successes with a number of recommendations based upon previous known lessons and reform programs.

ENVIRONMENTAL AND ECOSYSTEM CHALLENGES DUE TO SPILLAGE

Spillage has been known as the process of spilling or act of releasing liquid. However, when these liquid is released from an unfriendly industrial or domestic processes into the environment in such a quantity capable of causing environmental hazard, it becomes dangerous to the existing lives in the ecosystem. UNDP (2006) reported that most of the ecological degradation in oil-producing communities of the Niger Delta may be due to oil spillage, which could be as a result of accident linked to human error, equipment failure and deliberate destruction of oil pipes and pipelines. Approximately 6% of these spills were on land (UNDP, 2006) and other waste water sources also have inimical quantity of heavy metals and other environmentally unfriendly substances (Ademoroti, 1996; Afiukwa, 2013). Spillage from any source can contribute to land pollution. It is difficult to quantify or even summarize the effects of such pollution, since oils themselves vary much (Nelson, 2005). Frequent

crude oil spillage on agricultural soil, and the consequent fouling effect on all forms of life adversely affects the soils fertility such that most of the essential nutrients are no longer available for plant and crop utilization (Abii and Nwosu, 2009). However, plants germinate, develop and grow in soil medium where water, air and nutrient resources supply plants for healthy growth for productive and profitable agriculture (Essien and John, 2010); but in the case of land spillage, many researchers have reported its effects on plants growth. Udo and Fayemi (1995) reported that oil spillage accounted for 50% reduction in termination of *Zea mays* L. Ayesa et al. (2018) discussed extensively the poor growth of the seedlings of cash crop, *Dacryodes edulis* (African pear), hot pepper and tomato seeds, which are as a result of the suffocation of the plants due to exclusion of air by the oil that interfered with plants soil-water relationships. It has been estimated that oil concentration above 3% in the soil will reduce germination by suffocating seeds, thereby affecting their physiological activities (Amadi et al., 1996). Overall, spillage affected crop yield and farm income by extension of the social and economic livelihoods of farming communities (Odjuvwuederlie et al., 2006; Braide, 2000; Atubi and Onokala, 2006). Majority of agricultural and crop farming activities take place on the land including cotton farming; therefore, damages on the farmland due to spillage require close attention and revitalization technology. A conventional approach to solving this problem has been *ex-situ* but this is very expensive and damaging to the soil structure and ecology (Kramer et al., 2000); Salt and Kramer, 2000. Thus, an ecology and environment friendly approach (Phytoremediation) is reviewed.

SIGNIFICANCE OF PHYTOREMEDIATION

A shift to phytoremediation has become possible and necessary since it is environmentally and economically viable and very effective in the detoxification of contaminants (Yang et al., 2005). The main phytoremediation technologies are phytostabilization, phytoextraction, rhizodegradation and phytodegradation/ phytotransformation (Raskin et al., 1997; Salt et al., 1998; Nguyen et al., 2013; McCutcheon and Schnoor, 2003; Pilon-Smits and LeDuc, 2009). Phytoremediation is defined by UNEP (2012) as the use of living green plants for *in-situ* removal, degradation and containment of contaminants in soils, surface waters, and ground water. It offers a roadmap to increase the financial possibility of restoration programs, and to decrease disposal risks through the use of metal fortified plant biomass in energy and metal restoration with the burnt process. Phytoremediation is an emerging green technology that can be a promising solution to remediate hydrocarbon-polluted soils is not only in developed countries but also in developing countries like Nigeria, especially in the southern region. It is among the most potent and viable community-based management solutions for poor farmers. Based on phytoremediation versatility, it can also serve as useful link between researchers and farmers and will also recover the farming purpose of polluted soils. Phytoremediation application can be extended to anywhere pollution has affected the static water environment or an environment suffering from chronic danger due to pollution.

PLANTS PHYTOREMEDIATION MECHANISMS

Studies disclosed several species of plants have the ability to grow in contaminated soils and actually extract the pollutants through their roots system (Katherine, 1997). Heavy metals mostly exist in insoluble form in soil which is not bioavailable by releasing a variety of root exudates, which can change rhizosphere pH and increase heavy metal solubility (Dalvi and Bhaleroa, 2013); Gajic et al., 2018). There are series of processes involved in accumulation of heavy metals in plants, including heavy metal mobilization, root uptake, xylem loading, and root-to- shoot transport, cellular compartmentation and sequestration (Garba et al., 2011). Generally, there are two major ways plants can perform its function of phytoremediation: First is by bioaccumulating the pollutants in their tissues (Ndimele, 2003, 2010). The second is by converting the pollutants to less toxic components and then volatilizing them (Terry and Zayed, 1994; Brooks, 1998). Some of the end products are- alcohol, acids, carbondioxide and water, which are generally less toxic and less persistent in the environment than the parent compounds (Gordana et al., 2018). Transport of metal (loids) ions from roots to leaves is performed via membrane transporters, amino acids and/or organic acids (Jabeen et al., 2009). It is also known that fiber plants have many free hydroxyl groups at the molecular level that easily bond with oil or water (Bazargan et al., 2014), thus supporting the advancement of cotton for phytoremediation.

PHYTOREMEDIATION POTENTIAL PLANTS

Sunflower (*Helianthus annus* L.), an annual plant in the family Asteraceae has thus been identified as one of the target species that has great potential as a phytoextractor due to the fact that it produces large amounts of biomass, capable of hyper accumulating heavy metals in its harvestable parts (stems, leaves, and roots) and grows quickly (Francis, 2017; Nnamani and Nwosu, 2015)). In Nigeria, sunflower is one of the six common phytoremediation weeds (Wilberforce, 2015). Indian mustard (*Brassica jinxes* L.) is really useful to accumulate certain metals while producing high quantities of biomass in the process, and is the star of this group, as it can

remove three times more Cd than others, reduce 28% of Pb, up to 48% of Sc and can also remove Zn, Hg and Cu (Jay, 2015). White willow (*Salic* species) can deal with Cd, Ni and Pb, and even in mixed heavy metals such as dismal fuel polluted sites (Jay, 2015). Poplar tree (*Populous deltoids*), due to it naturally well-designed root system which take up large quantities of water can degrade petroleum hydrocarbons like benzene, toluene and D-xylene according to Canadian database. Indian grass (*Sorghastrum nutans*) is one of the nine members of Gramineae family identified by Phytopet as capable of remediating petroleum hydrocarbon. *Glycine max* L. Merr*, Zea mays* L., *Sorghum bicolor* (L.) Moench and *Medicago sativa* L. have been shown too to have phytoremediation potential. The most efficient crop of Cd, Pb, Cu, Ni, Cr, and Zn was *B. carinata* (Oksana et al., 2016). *Selaginella jacquemontii, Rumex hastatus,* and *Plectran thusrugosus* showed multifold enrichment factors (EF) of Fe, Mn, Cr, Ni, and Co (Muhammad et al., 2013). *Arabidopsis halleri* and *Solanum nigrum* have also been utilized for phytoremediation of cadmium (Ruchita et al., 2015). Typically, revegetation uses a combination of woody species and grasses. Woody species have included autumn olive (*Elaegnus angustifolia*), Scotch pine (*Pinus sylvestris*), red pine (*Pinus resinosa*), white pine (*Pinus strobus*), blafi locust (*Robinia pseudoacacia*), Virginia pine (*Pinu svirginiana*) and short leaf (*Pine echinata*). Pines tend to acidify soils which may increase mobility of heavy metals like Cd and Zn if they are present (Bergkvist et al., 1989). Paper birch (*Betula papyrifera*) has been shown to accumulate twice as much Cu as many other trees (Lepp and Dickson, 1998). David (2018) also disclosed *Petunia grandifloraJuss.* Mix F1 and *Marigold-Nemo* Mix have phytoremediation potentials. *Potamogeton natans* and *Alisma plantago-aquatica* were found to accumulate even higher concentration of Zn, Cu, and Pb (Fritioff and Greger, 2003). Furthermore, some other potential Nigerian weeds such as: *Phyllanthus amarus* (Chancapiedra), *Chromoloena odorate* (Awolowos weed), *Strachytarpheta indica* (Gervao), *Bryophyllum pinnatum* (Life leaf) and *Murraya koenigii* (Curry leaf) have been found to absorb Pb, Zn, Cd, Cu and Ni from contaminated sites (Nnabuk and Ndo, 2007). Guinea grass (*Panicum maximum*) and water leaf (*Talinum triangulare*) have shown phytoremediation potential for further clean-up of crude oil contaminated soil in Nigeria (Isaac, 2008). Waziri et al. (2016) also reported that jatropha (*Jatropha curcas*), neem (*Azadirachta ndica*) and baoba (*Adansonia digitata*) also have phytoremediation potential for heavy metals in Nigeria. Several researches within and outside Nigeria also reported several phytoremediation plants within and outside Nigeria. A few metals, including Cu, Mn and Zn, are however essentials to plant metabolism in trace amount. It is only when metals are present in bioavailable forms at excessive levels that they have the potential to become toxic to plants (Reichman, 2002).

HISTORY AND SUCCESS REPORT OF PHYTOREMEDIATION

Soil phytoremediation has only been developed in the last 30 years. A prolific literature of phytoremediation in the soil has been developed in that time (Brooks, 1998a; Mclntyre, 2003). Most plants that hyper accumulate metals have been identified for other areas than USA (Brooks, 1998). Like every other technology, phytoremediation can only be accepted if its success is demonstrated (Jean-Paul et al., 2002). Various chemical contaminants like suspended solids, dissolved oxygen, nitrogen, phosphorus and heavy metal etc. have minimized in India, China, Australia and Venezuela using phytoremediation plants (Roongtanakiat and Chairoj, 2001; Troung et al., 2008). Jean-Paul et al. (2002) also compared the success of phytoremediation in USA and Europe and several successes in many experimented sites were reported. The success of phytoremediation in USA paved way for the replication and advancement of the phytoremediation methods in other developing countries. These successes inspired integration of the approaches by Nigerian researchers towards actualizing similar results. However, minimal successes have been recorded in Nigeria using basically weeds and some other indigenous plants (Onyeike and Osuji, 2003). Cotton, thus, has not been integrated into this approach in Nigeria directly or by genetic modifications. Therefore, this limitation has formed the base of a further research.

PHYTOREMEDIATION BREAKTHROUGH WITH COTTON PLANT

Oil spillage in the Niger Delta region of Southern Nigeria has hindered cultivation of certain crops with less phytoremediation potential including cotton. Only 24 states in Nigeria are known for cotton farming (Nnaemeka and Sun, 2021) and none of the states is from the Niger Delta region. Success report of other plants for phytoremediation of polluted soils encouraged trial of economic plants for such purpose. Ramandeep et al. (2018) examined the bioaccumulation and translocation of heavy metals in different parts of cotton plant grown in an alkaline soil with very high sand contents which resulted in low retention of metals (As, Cr, Cu, Mn, Sr, and Zn). The bioaccumulation and translocation factors calculated for metal accumulation analysis in cotton plant parts were found above 1 (maximum 9.13 for Sr) indicating that cotton plant can become a significant system for phytoremediation. Cotton plant has a relatively large biomass, a profuse root system and capacity for heavy metal (Changfeng et al., 2020). Cotton as an economic fiber crop has a greater of becoming a promising candidate for phytoremediation because it could also minimize risk of human food chain contamination and ecological benefit. In this case, effort

towards improvement of cotton for this future position is recommended.

GENETIC MODIFICATION OF COTTON FOR PHYTOREMEDIATION

Plant breeding has been pursued to increase the efficacy of phytoremediation (Ernest, 2014). Some plants that are metal tolerant may not provide effective ground cover or have unknown cultural needs (Vangronsveld and Cunningham, 1998). Research has also been conducted in using transgenic plants for removal of mercury (Henry, 2000). In the last decade, there has been an increase in research on improving the ability of plants to remove environmental pollution (phytoremediation). Phytoremediation can be substantially improved using genetic engineering technology. Recent research results, including over expression of genes whose protein products are involved in metal uptake, transport or sequestration, or act as a small enzyme involved in the degradation of hazardous organic, have opened up new possibilities in phytoremediation (Sam and Margarida, 2005). Genes from microbes, plants, and animals are being used successfully to enhance the ability of plants to tolerate, remove, and degrade pollutants (Sharon, 2008). Sharon also reported, over expression of mammalian genes encoding cytochrome P450s led to increased metabolism and removal of a variety of organic pollutants and herbicides. Gisbert et al. (2003) produced a tobacco plant that could remove more lead from soil than normal tobacco plant by inserting a gene from wheat plants that produces phytochelatin synthase into a shrub tobacco plant (*Nicotiana glauca*) to increase its absorption and tolerance of toxic metals particularly leaf. They found that the genetically modified plants absorbed about twice as much lead compared to non-modified plants. S-transferase has been introduced into higher plants, resulting in significant improvement of tolerance, removal and degradation of pollutants (Benoit and Sharon, 2009). Sharon (2008) has also recognized plant associated bacteria playing a significant role in phytoremediation leading to the development of genetically modified rhizospheric and endophytic bacteria. So far, several evidences have supported interest in adding such or similar genes from its parent organisms into cotton plant in order to improve cotton phytoremediation strength and pave way for future hope in cotton farming amidst pollution. Research involving cotton plant in this advance technology is insufficient. Transgenic plant technology for remediation of toxic metals and metaloids covers all the technical aspect of gene transfer from molecular methods to field performance using a wide range of plants and diverse abiotic stress factor (Majeti, 2019). In other words, the methods for the modification of cotton plant will also follow standard molecular methods.

CONCLUSION

Cotton is grown in around 105 countries with about 10 countries contributing highest quantity (Figure 1). Africa as a whole contributes less than 5% to the global demand for cotton and Nigeria which used to be Africa's leading cotton producer and 12th largest in the world now accounts for about 20.29% of Africa's cotton production by 2029. However as at 2020, Nigeria's share of Africa cotton production stood at 29.28% compared to Africa's projected cotton production share in 2029 which is expected to decline by about 7.60% (OECD-FAO Agricultural Outlook, 2020). These downtrends have been since 2010. With a total production capacity of 602,440 metric tons, Nigeria was Africa's leading cotton producer but declined to 51,000 metric tons in 2020. Also, export earnings from cotton plunged too significantly to about #866 million in the third quarter of 2020 from #1.71 billion in 2010. Efforts towards reviving the production and earnings have become paramount. Therefore, increasing the production capacity of cotton by introducing cotton farming in other areas of Nigeria beyond the Savanna belt region where they are currently grown formed the base of this review. To actualize this, employing genetic engineering approaches by developing genetically modified cottons capable of surviving and yielding in the Niger Delta region despite the polluted soils is recommended. Nevertheless, native plant species grown in particular areas over a long period of time without human intervention possess certain characteristics that make them the best adapted to local conditions, providing practical and ecologically valuable alternative for landscaping and eco restoration projects (Chidi et al., 2015; Dorner, 2002). Therefore, phytomanagement of degraded sites encourages selection of native species that in the long run form self-sustaining plant communities that do not require much maintenance (Gordana et al., 2018). In spite of the successes already recorded, dependence on the natural plants with phytoremediation potential may not meet the future demand for healthy environment as a result of increase in industrialization and urbanization from which wastes are discharged. Thus, advance in use of genetic approaches towards modifying naturally none or less phytoremediation potential plants with high cultivation and economic demand is necessary. This recommendation is possible because current trend of cotton breeding have been successful and these techniques have increased the productivity of cotton within the period of invention. With the advancement in cotton genomics, some important genomes and genomics banks have been constructed. Also, newer wild species have been discovered and many countries are conserving genetic resources within and between species, implying that this valuable germplasm can be exchanged among countries for increasing productivity (Iswarappa et al., 2020). There is huge scope for pre

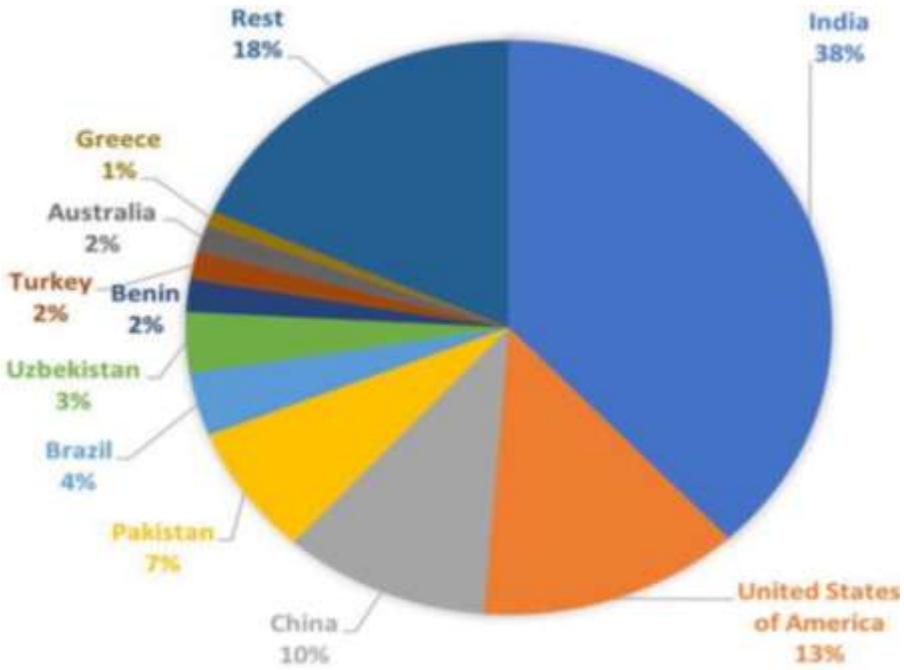

Figure 1. Highest cotton-producing countries.
Source: Ishwarappa et al. (2020).

Table 1. Countries with wide list of cotton germplasm.

Country	*G. hirsutum*	*G. barbadense*	*G. herbaceum*	*G. arboreum*
India	8851	536	565	2053
Uzbekistan	13,241	3019	1495	1185
United States of America	6302	1584	194	1729
China	7752	633	18	433
Russia	4503	1057	336	365
Brazil	1660	1509	19	219
France	2173	483	50	69
Australia	1573	99	39	211

Source: Ishwarappa et al. (2020).

breeding work in cotton to combat biotic and abiotic stresses. Some countries however, have maintained wide list of cotton germplasm (Table 1), supporting the possibilities of transforming cotton to combat abiotic stress due to pollution in Southern Nigeria.

Based on this, interest in the use of transgenic cotton as one of the most promising candidates for phytoremediation is recommended. In this regard, it becomes essential to select upland cotton (*G. hirsutum* L.) for transformation with genes known to influence phytoremediation in plants. The sole aim is to support initiation of research into development of transgenic cottons with high phytoremediation strength and results are expected to be integrated in the agriculture and environmental advancement strategies.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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