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Full Length Research Paper

Expression profiling, phylogenetic, and structural analyses of a laccase gene from the red palm weevil, *Rhynchophorus ferrugineus*

Babiker M. A. Abdel-Banat* and Hamadttu A. F. El-Shafie

Date Palm Research Center of Excellence, King Faisal University, Al-Hofuf, Al-Ahsa 31982, Saudi Arabia.

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Laccases, member of multicopper oxidase (MCO) family enzymes, play crucial roles in insects' cuticle tanning and pigmentation. The purpose of this study was to identify and characterize a laccase gene from the red palm weevil (RPW), *Rhynchophorus ferrugineus*. The isolated RPW laccase gene sequence was 3,389 bp, including a 2,163 bp open reading frame that encodes 720 amino acids. The RPW laccase gene conserved the MCO functional motifs Type-3, Type-1, and Type-2, respectively. Phylogenetic analysis categorized this protein into the functional cluster 2 of insect laccases (*Lac2*). The primary transcripts for *R. ferrugineus* laccase 2 (*RfeLac2*) were highly expressed in the adult's cuticle, elytra, and hindwings, and in the larval cuticles four days before molting. Then, the transcripts were declined drastically in the larval cuticles three days before molting. This implies that the suppression of *RfeLac2* in the larvae occurs earlier than expected. *RfeLac2* transcripts were very low in the gut and adipose tissues of larvae and adults, irrespective to the span to undergo molting. This suggests that *RfeLac2* is not active in the tissues that do not undergo heavy sclerotization. Three-dimensional (3-D) structure modeling of *RfeLac2* predicted eight histidines, one glycine, and one phenylalanine, as copper-binding ligands on the laccase active center. The study finding indicates that the pattern of the RPW *RfeLac2* expression varies from other coleopteran insects, a phenomenon that requires further investigation.

Key words: Cuticle, expression, laccase, phylogeny, Red palm weevil.

INTRODUCTION

Laccases (EC 1.10.3.2) are metalloenzymes that belong to the multicopper oxidase (MCO) family (Ye et al., 2015). These enzymes catalyze the oxidation of various aromatic substrates with simultaneous reduction of molecular oxygen to water. They lack the monooxygenase activity, but they are able to oxidize *ortho*- and *para*-phenols, meanwhile they catalyze the oxidation of polyphenols, diamines, substituted phenols, and aromatic amines (Riva, 2006). A typical laccase active center contains four copper atoms and ten highly conserved histidines (Shi et al., 2017). Laccases are widely distributed in nature and with broad physiological functions depending on both their origin and on their biochemical and structural properties (Cazares-Garcia et al., 2013). They are present in bacteria, plants, fungi, insects, and marine invertebrates. They function in

*Corresponding author. E-mail: babikera@hotmail.com; bahmed@kfu.edu.sa. Tel: +966 13 589 8749. Fax: +966 13 589 7243.

pigmentation, lignin synthesis and degradation, iron homeostasis, sporulation, rhizomorph formation, morphogenesis, and immune defense (Dittmer and Kanost, 2010; Shi et al., 2014). This immense functional versatility is partly because laccases possess low substrate specificity and exhibit a broad range of redox potentials (Giardina et al., 2010).

Several laccase isoforms have been identified and characterized in insects and have been suggested to be involved in cuticle sclerotization and pigmentation (Hattori et al., 2005, 2010; Coy et al., 2010; Yatsu and Asano, 2009; Arakane et al., 2005; Dittmer et al., 2004; Futahashi et al., 2011; Yang et al., 2017). There are two main isoforms of insect laccases identified so far. One of them (laccase 2) has been proven to be involved in sclerotization and pigmentation of cuticles of the red flour beetle *Tribolium castaneum TcLac2* and the pine sawyer *Monochamus alternatus MaLac2* by using RNA interference (RNAi) (Arakane et al., 2005; Niu et al., 2008). Laccase 2 of the mosquito *Culex pipiens pallens CppLac2* was found to induce heavy sclerotization of the cuticle, which could reduce insecticide penetration and thus confer insecticide resistance because a higher level of *CppLac2* mRNA was observed in the insecticide-resistant populations (Pan et al., 2009). The other isoform is laccase 1, which was expressed in the midgut, Malpighian tubules, and fat body as well as in the epidermis of the tobacco hornworm *Manduca sexta*. It may function to oxidize toxic compounds ingested by the insect (Dittmer et al., 2004). There are salivary gland laccases identified in the green rice leafhopper *Nephotettix cincticeps* (Uhler) *NcLac1S* and the whitefly *Bemisia tabaci* MED *BtLac1*. These possibly function in the detoxification of plant phenolic compounds and coagulation of the salivary sheath during feeding (Hattori et al., 2010; Yang et al., 2017). Phylogenetic analysis of genes from seven insect species belonging to four orders led to the identification of putative orthologs of MCO1 (Lac1) and MCO2 (Lac2) in all of the insect genomes examined (Gorman et al., 2008). Whereas, MCO3 (Lac3), MCO4 (Lac4) and MCO5 (Lac5) were found only in *Anopheles gambiae* and other species of mosquito, such as *Aedes aegypti*. The genes in this mosquito-specific cluster share a common ancestor with MCO2 (Gorman et al., 2008).

The red palm weevil (RPW), *Rhynchophorus ferrugineus*, is an invasive and globally important quarantine pest of palm trees. The weevil was introduced to Saudi Arabia from Southeast Asia during the 1980s. It subsequently spread to all Middle East countries and has since migrated into Spain and Southern France (Dembilio and Jaques, 2015; Al-Dosary et al., 2016). Food and Agriculture Organization of the United Nations (FAO) has classified the RPW as category-1 pest on date palm in the Middle East (Al-Dosary et al., 2016). The weevil completes its entire larval life cycle within the palm trunk, which renders detection of its early infestation difficult and its control with the conventional methods have evidenced unsuccessful (Faleiro et al., 2012; Hoddle et al., 2013). Looking for possible alternative control methods, thus, synthetic biology approaches were proposed. Such as disruption of pheromone communication machinery that interrupts the weevil's olfaction to find host and mate, and finally interrupting its reproduction leading to population decline (Antony et al., 2016, 2018; Soffan et al., 2016). Additionally, cuticular proteins such as laccases and others also proposed as important targets for disruption since they function in cuticle hardening to protect insects from environmental stress and mechanical damage. Understanding the structure of the functional motifs of the laccase gene from the RPW and elucidation of its phylogenetic relationship to laccases from other insect species will help to formulate tactics to the utilization of these motifs for further studies aiming at the gene disruption. Therefore, the objective of this study was to isolate laccase gene from the RPW, analyze its functional motifs, and to study its expression profile in the tissues of different developmental stages of the RPW.

MATERIALS AND METHODS

RPW rearing and tissue collection

The RPW, at all developmental stages, was reared in the date palm research center facilities as described previously (Abdel-Banat et al., 2018; El-Shafie et al., 2013). For the purpose of egg-laying, male and female adults were fed on sugarcane kept in TATAY storage boxes (51 cm × 38 cm × 26 cm) with perforated lids. The boxes are made of polypropylene and bisphenol A (BPA) free (www.tatay.com). Eggs were removed from the sugarcane with a brush and placed in Petri dishes that contained cotton and moist filter paper and incubated at 28°C until the eggs hatch. First instar larvae were collected daily and reared on pineapples or date palm trunk. Samples of different developmental stages were collected periodically for the integument and other tissues collection. Larvae were dissected by cutting off their heads using a standard stainless steel entomology dissection set. The integument was cut longitudinally to separate the adipose tissues and the guts. The dissected tissues were immediately frozen in liquid nitrogen. Eggs, elytra, forewings, and the adult's body were directly frozen in liquid nitrogen. All samples were stored at -80°C for the subsequent experiments.

BLAST search and sequence alignment

The online Basic Local Alignment Search Tool (BLAST®) was used to search for potential laccase gene sequences in the RPW Transcriptome Shotgun Assembly (TSA) (Wang et al., 2013; Antony et al., 2016). *T. castaneum* and other insect's laccase gene sequences that are available in the NCBI GenBank® were used to search for similar sequences in the RPW TSA dataset. The identified RPW TSA sequences were pools of unannotated sequences with gaps in the sequenced contigs. Multiple sequence alignment was done using MEGA X (Kumar et al., 2018) software in order to locate the highly conserved signature sequences in the amino acids of known insect laccases. Only RPW TSA contigs that show highly conserved sequences of multicopper oxidase, namely

Table 1. Primers used for *RfeLac2* cDNA synthesis and cloning, sequencing, and semi-quantitative RT-PCR analysis.

Primer name	Sequence (5′→3′)	Purpose
RfLac-1	GCCAAATTTTTTCAGCAGCAGCGCGGTAATA	Full cDNA; RT-PCR
RfLac-2	GAAGATGGACGGCATCTACGGCAGCATC	Sequencing
RfLac-3c	GCATCCAATCGGACAGGAGGATGACGTG	RT-PCR; Sequencing
RfLac-4	CACTTATAACAGGCATTTAGTTGCTCCA	Sequencing
RfLac-5c	GGTGCACATACAGTTGGGACCACAGTCG	Sequencing
RfLac-6	CTATCTTTCGGTGCCATCGGTCTCGGTC	Sequencing
RfLac-7c	GGAGACTGGCGTTGACGCGTCCAAGGAT	Sequencing
RfLac-8c	CTCTTTACAATAATAGAACATCGAAGGAGT	Sequencing
RfLac-9c	ATACATAAATTATAATTTTATTTCTATATCCA	Full cDNA; Sequencing

Types-3, -1, and -2 motifs, were used to synthesize the primers (Table 1), which have been used for amplification of the RPW laccase gene.

RNA isolation and first-strand cDNA synthesis

Frozen tissues from individual RPW larva were ground into fine powder in liquid nitrogen using mortar and pestle. Total RNA was isolated from 50 mg larval and adult tissues using RNeasy Plus Universal Mini Kit (QIAGEN) according to the manufacturer's protocol. The total RNA concentration was measured using NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific). Elongase™ enzyme mix was obtained from Invitrogen®. Primers used to amplify the full-length cDNA of laccase, to study its expression pattern, and those for sequencing purpose are shown in Table 1. Reverse transcription of RNA to synthesize the first-strand cDNA for laccase was done using RevertAid RT Kit according to the manufacturer's protocol (Thermo Fisher Scientific). Briefly, 0.5 µg total RNA, 100 µM random hexamer primer, 5× reaction buffer, 20 U RiboLock RNase inhibitor, 20 mM dNTP mix, 200 U RevertAid RT, and nuclease-free H_2O were mixed in a total reaction volume of 20 µl. The mixture was incubated at 25°C for 5 min then followed by 60 min at 45°C. The reaction was terminated by heating at 70°C for 5 min. Double-stranded cDNA was amplified in a total volume of 50 µl using 2 µl from the first-strand cDNA reaction as template, pair of gene-specific primers (10 pmol/µl each), 200 µM each dNTP, 1 µl Elongase™ enzyme mix, and 1.8 mM final [Mg^{2+}]. The thermocycling program was as follows: One cycle for initial denaturation at 94°C for 3 min, followed by 35 cycles for denaturation at 94°C for 2 s, annealing at 57°C for 25 s, and extension at 68°C for 6 min. The program was ended with a final extension cycle for 10 min at 68°C. The thermocycler used for cDNA synthesis and the subsequence amplifications was Veriti® Thermal Cycler (96 well) supplied by Applied Biosystems™. Amplified PCR products were electrophoresed on 0.7% agarose D1 (Pronadisa) gel, stained with ethidium bromide, visualized using INGENIUS Syngene Bio Imaging System, and documented using GeneSnap software from Syngene. Then, the cDNA was purified either from the excised gel using QIAquick® Gel extraction kit (QIAGEN) or directly from the PCR products using the DNA Pure Kit (Geneaid®) following the manufacturers' protocols. The recovered cDNA was used for the subsequent PCR amplification, cloning, or direct sequencing.

Gene cloning and sequencing

The PCR-amplified cDNA was cloned into the pGEM®-T Easy vector (Promega, Madison, WI, USA) according to the manufacturer's protocol. Ligation, cloning, and transformation processes were done according to the standard protocols (Sambrook and Russell, 2001). The manipulated plasmids were transformed into *Escherichia coli* strain DH5α. Plasmids maintained by the bacterium were isolated using Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA) according to the supplier's instructions. Multiple sequencing rounds were done to clarify the dubious and to read long uncovered sequences. Sequencing was performed at Macrogen service facilities (Seoul, South Korea).

Sequence and domain structure analyses

The deduced amino acid sequences of isolated laccase were analyzed and compared with insect laccases using the BLAST algorithm. Prediction of the signal peptide and the cleavage site was performed with the program SignalP server (http://www.cbs.dtu.dk/services/SignalP-3.0/) (Bendtsen et al., 2004). Phyre2, a protein fold recognition server (Kelley et al., 2015), 3DLigandSite server for ligands prediction (Wass et al., 2010), and InterPro, a protein sequence analysis and classification database server (Mitchell et al., 2019) were used to predict the RPW laccase 3-D structural modeling, ligand-binding sites, and the laccase signature domain structure.

Phylogenetic analysis

Multiple sequence alignment of the deduced amino acids of laccases and the phylogenetic analyses were performed using the software MEGA X (Kumar et al., 2018). Sequences were aligned using ClustralW program integral to the MEGA X software and the phylogenetic tree was constructed by the neighbor-joining method. This analysis involved 104 amino acid sequences from 70 insect species belonging to seven orders. More than five trees were constructed using these sequences on the same program. The sequences (with GenBank® accession numbers) used for the analysis were listed in Supplementary Table 1.

Expression profile of laccase in different developmental stages

The expression profiles of the RPW laccase gene at larval and adult developmental stages were analyzed (Abdel-Banat et al., 2018). Tissues of the middle-aged larvae were collected four days to one day before molting. Adult cuticles and gut tissues as well as forewings (elytra) and hindwings were also used for the laccase

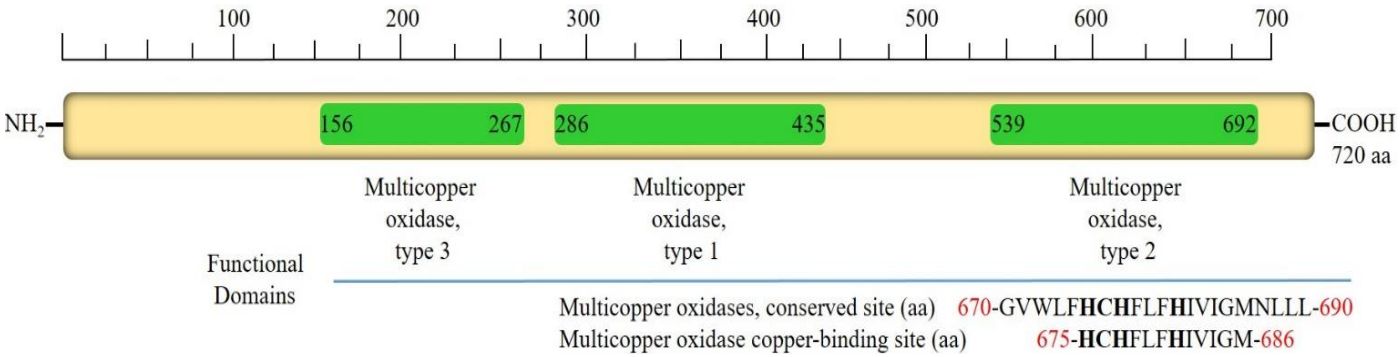

Figure 1. Domain organization of putative RPW *RfeLac2*. The gene encodes 720 amino acids. Functional motifs of MCO Type-3, MCO Type-1, and MCO Type-2 were depicted relative to their positions within the sequence.

expression analysis. Reverse transcription PCR for the laccase gene and the *Rfeβ-actin1* internal reference gene was done in a 25 µl reaction mixtures containing 1 µl template from the first-strand cDNA synthesized from 0.5 µg total RNA, 10 pmol/µl each gene-specific primer, 12.5 µl Master Mix (Biomatik Corporation, Canada), and nuclease-free water. The thermocycler program for RT-PCR was as follows: Initial denaturation cycle at 94°C for 3 min followed by 30 cycles at 94°C for 25 s, 60°C for 25 sec, and 72°C for 2 min, and a final extension cycle at 72°C for 10 min. PCR products were analyzed on 1% agarose gel. Experiments for RT-PCR were replicated at least three times using independent total RNA preparations.

RESULTS

Molecular characterization of *RfeLac2*

A cDNA clone of a laccase gene was isolated from the RPW and the sequence was identified and deposited at the GenBank® database with the accession number (MK655469). The complete mRNA consists of 3,389 base pairs (bp). The open reading frame is 2,163 bp, which encodes a putative protein of 720 amino acids (aa) including a predicted N-terminal signal peptide of 21 aa. Three conserved MCO motifs were found in the putative sequence of *RfeLac2* protein. The amino acids from 156 to 267, from 286 to 435, and from 539 to 692 (Figure 1) exemplify the motifs of MCO Type-3, MCO Type-1, and MCO Type-2, respectively. Within those conserved regions, there are eight histidines (His203, His205, His247, His249, His601, His603, His673, and His675), one glycine (Gly206), one phenylalanine (Phe245), and one cysteine (Cys674) (Figure 2). The sequence $_{111}$-C~C-$_{115}$ at the N-terminal region of *RfeLac2* represents the insect-specific (C-X-R-X-C) sequence commonly found in all identified insect laccases (where X represents any residue). Downstream to the insect-specific sequence is a sequence composed of 24 amino acids ($_{203}$-HWHGIWQKGSQYYDGVPFVTQCPI-$_{226}$) that contain consensus sequences in all laccases (underlined).

A phylogenetic tree was constructed by the neighbor-joining method from 104 amino acid sequences compiled from the GenBank® database to compare with *RfeLac2* (Figure 3). The sequences represent 70 insect species from seven orders (Supplementary Table 1). In general, the phylogenetic tree showed clustering of laccases according to the orders of insects. *RfeLac2* was clustered into the group of insect laccase 2 that includes, for instance, *T. castaneum TcaLac2*, *M. alternatus MalLac2*, *Chrysomela populi CpoLac2*, and *Phaedon cochleariae PcoLac2*. However, the *C. pipiens pallens CppLac2* was clustered in the dipterous laccases clade and the *N. cincticeps NciLac2* was clustered in the hemipterans clade. Whereas group 1 laccases (Lac1) from various insects including *T. castaneum TcaLac1*, *A. gambiae AgaLac1*, *M. sexta MseLac1*, *N. cincticeps NciLac1G* and *NciLac1S*, *Acyrthosiphon pisum ApiLac1*, *B. tabaci BtaLac1*, and *Apis mellifera AmeLac1*, in addition to MCO1 from *Drosophila melanogaster DmeMCO1A*), *Helicoverpa armigera HarMCO1*, and *Bombyx mori BmoMCO1* were clustered together on the phylogenetic tree.

Expression patterns of *RfeLac2*

During and immediately after molting the beetle's exoskeleton and mouthparts are soft and fragile, thus, throughout this period the insect stops feeding. Therefore, adult or larva starts feeding soon after sufficient cuticle sclerotization and the body maintains its physical strength. There is a clear variation in *RfeLac2* transcripts expression pattern in the larval tissues and the adult's body parts (Figure 4). A strong expression level of *RfeLac2* was found in the elytra (forewings), hindwings, and the adult's cuticle, but a weak expression level of the gene was observed in the adult's gut. It is noticeably high *RfeLac2* transcripts in the larval cuticles were observed four days before the start of molting, but very little or hardly detectable transcripts were observed in the gut and fat body of the larvae investigated during the same period. Remarkably, *RfeLac2* transcripts were decreased drastically in all larval tissues three days before the onset

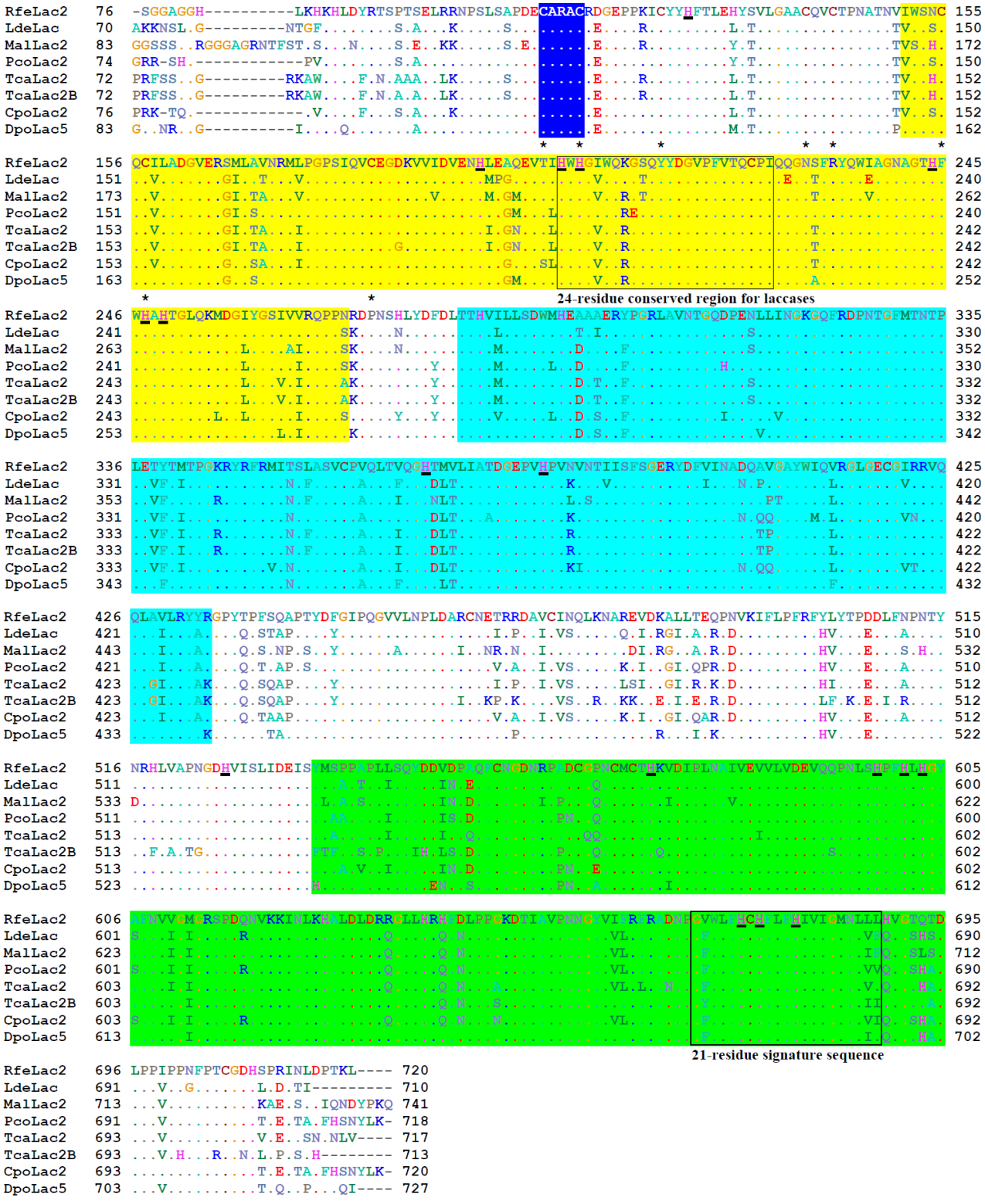

Figure 2. Multiple sequence alignment of the deduced amino acids of laccase 2 genes (*Lac2*). Sequences from eight coleopteran insects were aligned. The insect laccase-specific sequence C-X-R-X-C is highlighted in blue. Asterisks below the sequences indicate cysteine residues conserved at N-terminal in all laccases. MCO Type-3 sequence is highlighted in yellow, Type-1 is highlighted in turquoise, and Type-2 is highlighted in green. The 24-residue conserved region at Type-3 and the 21-residue signature sequence at Type-2 ligand binding sites are boxed. Conserved histidine residues are underlined. *Rfe, Rhynchophorus ferrugineus; Lde, Leptinotarsa decemlineata; Mal, Monochamus alternatus; Pco, Phaedon cochleariae; Tca, Tribolium castaneum; Cpo, Chrysomela populi; Dpo, Dendroctonus ponderosae*.

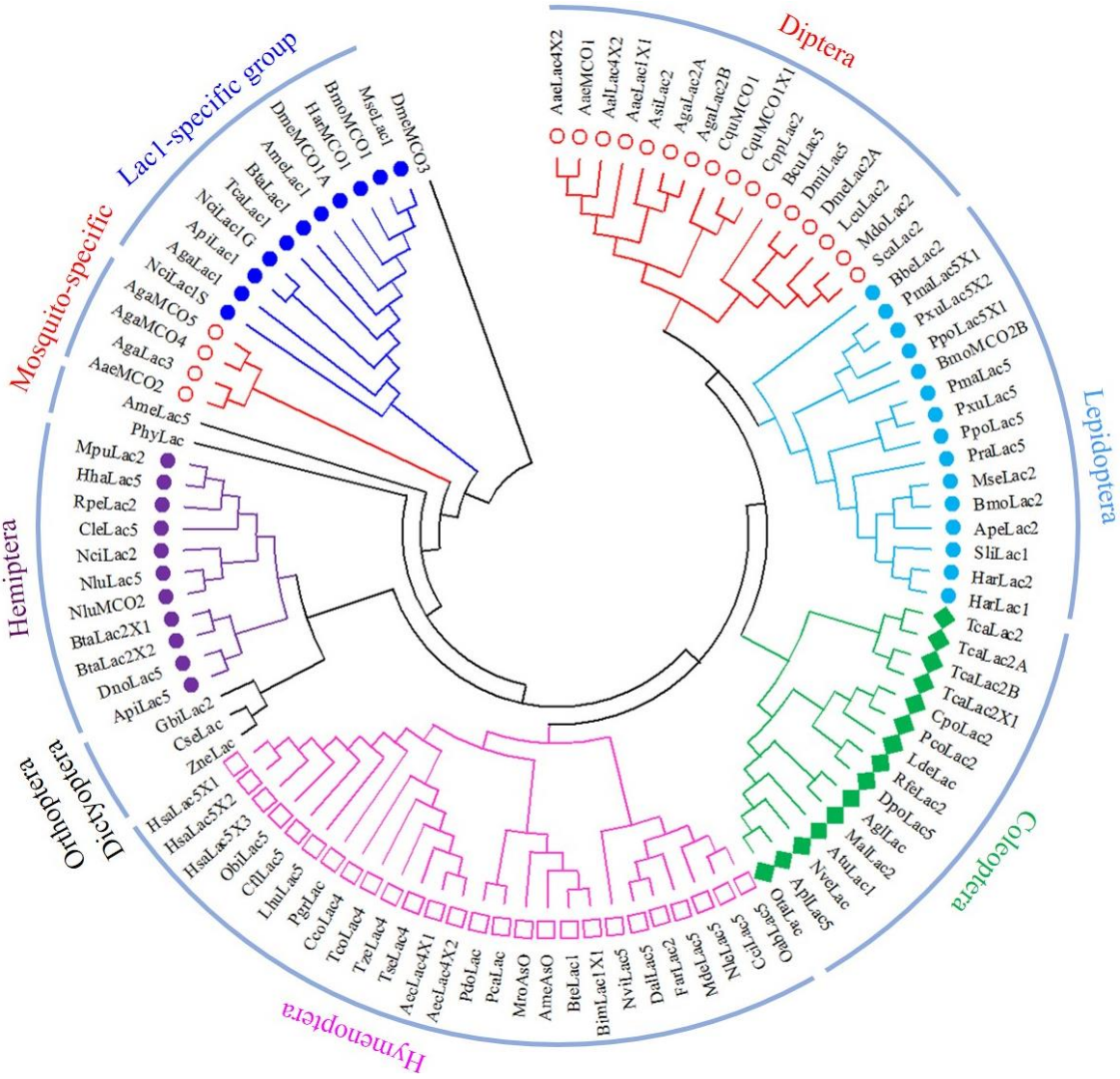

Figure 3. Phylogenetic analysis of insect laccases (Lac) and multicopper oxidases (MCO). The tree was constructed from the deduced amino acid sequences of 104 genes by the Neighbor-Joining method implemented in MEGA X. The evolutionary distances were computed using the Poisson correction method integral to MEGA X software and are in the units of the number of amino acid substitutions per site. The genes that used to construct this tree and their accession numbers are provided in details as Supplementary Table 1.

of molting (Figure 4).

Prediction of *RfeLac2* three-dimensional structure and ligand binding

Three-dimensional (3-D) structure of *RfeLac2* was predicted by the homology modeling approach. Templates used for the prediction were c3ppsD, c3sqrA, c2q9oA, c1zpuE, and c1gycA. Ligands found in the predicted binding site are eight histidine residues, one glycine, and one phenylalanine (Figre 5). The *RfeLac2* Type-3 (residues 156 to 267) and Type-2 (residues from 539 to 692) copper centers were analyzed separately to predict the 3-D structures for these centers. Predicted ligands at Type-3 copper center were four histidine residues, one glycine, and one phenylalanine and those predicted at Type-2 center were four histidine residues (Figure 5). No ligand was predicted for *RfeLac2* Type-1 copper center when analyzed separately (data not shown).

DISCUSSION

In this study, the RPW laccase 2 (*RfeLac2*) cDNA was

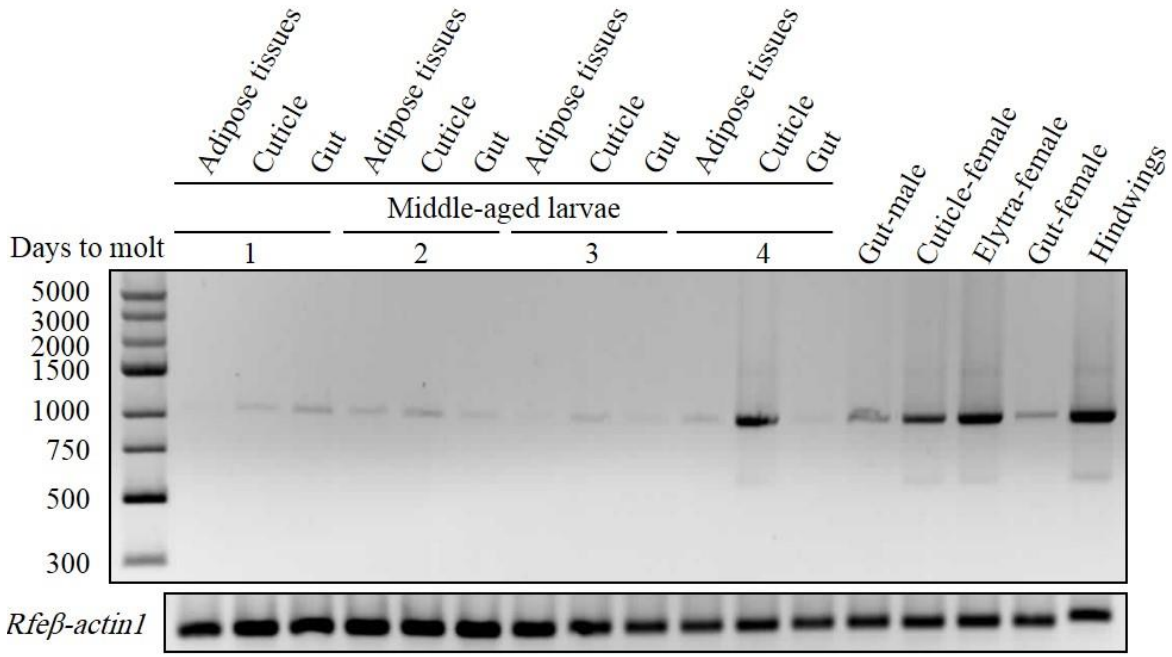

Figure 4. *RfeLac2* expression patterns in the larval and adults tissues. The expression of *RfeLac2* gene was evaluated four- to one-day pre-molting in the adipose tissues, cuticles, and guts of the middle-aged larvae. Likewise, the gene's expression level was investigated in the adult's cuticle, gut, elytra, and hindwings. *Rfeβ-actin1* was used as a reference gene for the RT-PCR.

isolated and its entire sequence was identified. The deduced amino acid sequence of *RfeLac2* shows high identity to those of other insect *laccase 2* genes, particularly to *Lac2* genes of *T. castaneum TcaLac2* (Arakane et al., 2005; Julio et al., 2017), *C. populi CpoLac2* and *P. cochleariae PcoLac2* (Pentzold et al., 2018), *Leptinotarsa decemlineata LdeLac* (Clements et al., 2016), and *M. alternatus MalLac2* (Niu et al., 2008). It also shows high identity to the laccases predicted by automated computational analysis of the genomic sequences of *Dendroctonus ponderosae* and *Anoplophora glabripennis*. The deduced protein from *RfeLac2* contains the MCO conserved regions as described in many insect laccases (Dittmer and Kanost, 2010). The consensus sequence for insect laccases has been defined as HWHG-$(X)_9$-DGVP-$(X)_3$-QCPI, whereas the consensus sequences for fungal and plant laccases have been defined as HWHG-$(X)_9$-DG-$(X)_5$-QCPI and HWHG-$(X)_9$-DGP-$(X)_3$-TQCPI, respectively (Kumar et al., 2003). The first and the last four underlined amino acids of the consensus sequence were common in laccases of insects, fungi, and plants. The sequence ($_{673}$-HCHFLFHIVIGM-$_{684}$) at the C-terminal of *RfeLac2* was conserved in all insect laccases. It is twelve amino acids long representative of Type-2 copper oxidase signature and the same consensus sequence has been defined in fungi as HCH-$(X)_3$-H-$(X)_3$-[A/G]-[L/M] (Kumar et al., 2003). The core of this consensus motif is three histidines and one cysteine.

Phylogenetic analysis of putative insect laccases from 70 species has shown clustering of these proteins according to their respective insect orders with some exceptions. The coleopteran laccase 2 proteins, including *T. castaneum TcaLac2* (Arakane et al., 2005; Jacobs et al., 2015; Julio et al., 2017)*, M. alternatus MalLac2* (Niu et al., 2008)*, Chrysomela populi CpoLac2*, and *Phaedon cochleariae PcoLac2* (Pentzold et al., 2018), were grouped into one clade together with the currently investigated *RefeLac2*. Moreover, the dipterous and hemipterans laccase 2 proteins were grouped into their respective orders despite the fact that many of them have been proven to function in hardening of cuticles, proper morphology, and pigmentation (Pan et al., 2009; Hattori et al., 2010) as do the coleopteran laccase 2. The phylogenetic tree also showed an interesting feature for most functionally characterized insects' laccase 1. This suggests that they probably share common functional properties. The branch of the laccase1-specific group includes laccase 1 and MCO1 proteins from five orders namely Hemiptera, Lepidoptera, Diptera, Hymenoptera, and Coleoptera. This group of enzymes was found to function in the detoxification of secondary plant compounds (Yang et al., 2017) and in iron homeostasis (Dittmer et al., 2004; Lang et al., 2012; Liu et al., 2015). It is notable that the group of MCOs previously described as mosquito-specific (*AgaMCO3-5* and *AaeMCO2*) (Dittmer and Kanost, 2010) was clustered together in a separate branch of the current phylogenetic tree.

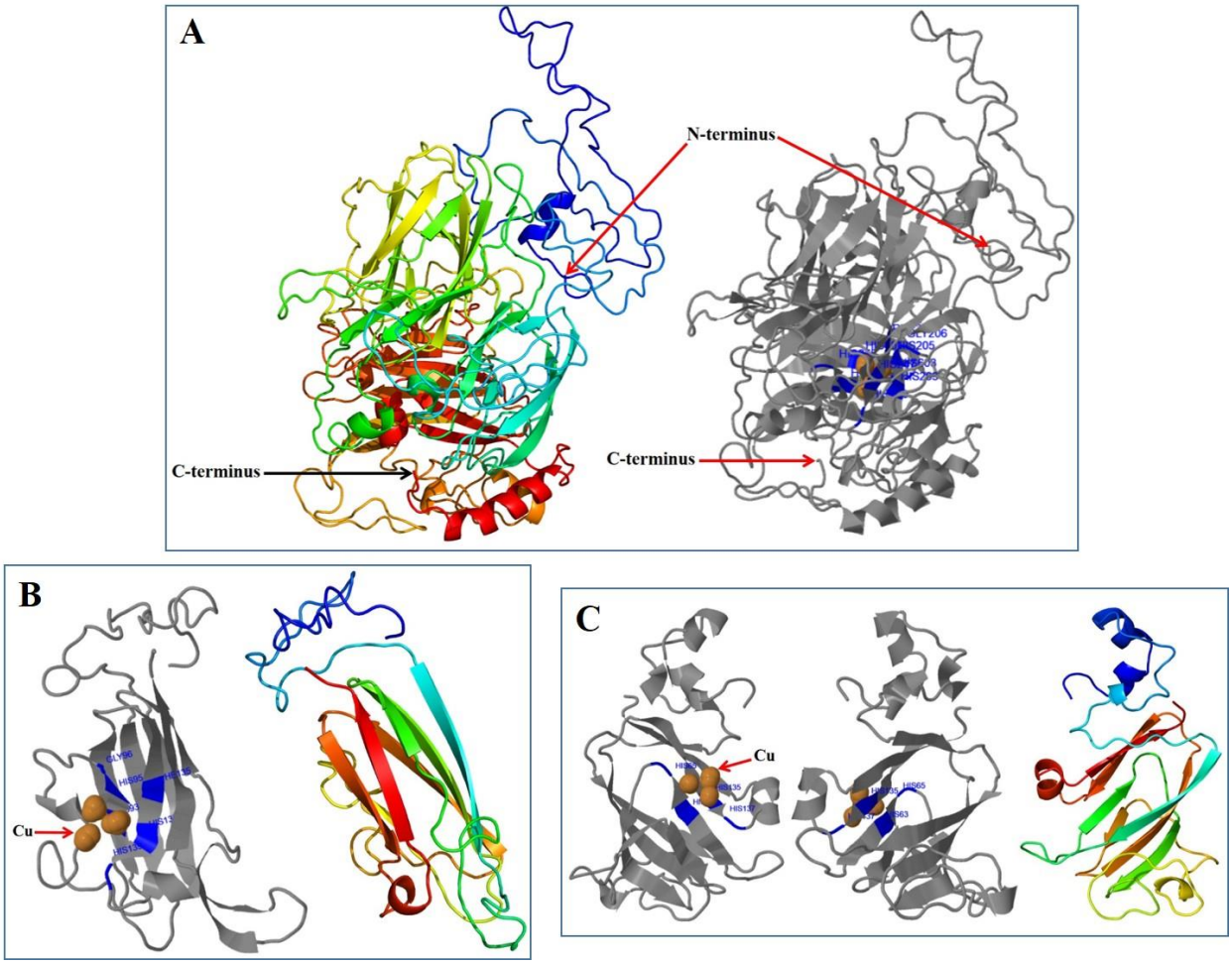

Figure 5. Three-dimensional (3-D) structure of *RfeLac2* and the copper-binding ligands. (**A**) Predicted 3-D structure of *RfeLac2.* Copper appears in the predicted binding site. Ligands in the predicted binding site are eight histidine residues, one glycine, and one phenylalanine (right panel). (**B**) Predicted 3-D structure of multicopper oxidase Type-3 domain of *RfeLac2* (residues 111 to 267) and (**C**) Predicted structure of multicopper oxidase Type-2 domain of *RfeLac2* (residues 539 to 692).

The *RfeLac2* primary transcripts are most abundant in hindwings, elytra, and the cuticle of adult weevil as well as in the cuticle of larvae examined four days before molting. The expression is very low in the adult gut and sharply declined in the cuticle of larvae examined one to three days before molting. Similar expression pattern was observed in the stinkbug, *Riptortus pedestris* (Hemiptera: Alydidae) *RpeLac2* (Futahashi et al., 2011). Thus, the findings from these two studies highlight the expression of *Lac2* peaks days before the larval molting in both species. Contrary to the expression patterns of *RfeLac2* and *RpeLac2*, are the studies, for instance, on *T. castaneum*, *M. sexta*, *B. mori*, and *M. alternatus*, in which the expression of *Lac2* was reported in the epidermis just prior to larval molt (Yatsu and Asano, 2009; Dittmer et al., 2004; Niu et al., 2008). Therefore, the opposite expression patterns of *RfeLac2* and *RpeLac2* relative to the other species, particularly in the larval cuticle, could be attributed to the broad span of the examined samples from the two studies. The expression pattern of the RPW *RfeLac2* suggests that this enzyme promotes the larval cuticle sclerotization, together with other proteins, and its activity gradually diminishes as the larva approach molting in order to facilitate the de-sclerotization of the cuticle making it amenable to the subsequent degrading enzymes.

The *RfeLac2* 3-D structure was predicted on the basis of the topology of the crystal structure of an ascomycete fungal laccases from *Thielavia arenaria* (Kallio et al., 2011) and from *Botrytis aclada* (Osipov et al., 2014), since no crystal structure for insect laccase is available to date. The analysis predicted the basic topology for *RfeLac2*, the copper active centers, and the ligands that bind copper during enzymatic catalysis. Eight histidine residues, a glycine and phenylalanine residues appeared as copper ligands, but the conserved residue cysteine

does not appear as a ligand on the predicted 3-D structure of *RfeLac2*. It was proposed that residues that do not ligate with copper ions were either conserved or semi-conserved to maintain a local 3-D fold (Kumar et al., 2003). This observation might be common to many laccases due to the hidden features of laccases that are not clear by comparison of the amino acid sequences alone or by comparison of the 3-D structures alone (Kumar et al., 2003). It has been reported that Type-1 copper center shows coordination with two histidines, one cysteine, and one methionine as ligands. The Type-2 copper has two histidines and water as ligands. The Type-3 copper coordination with three histidines and a hydroxyl bridge, maintains the strong anti-ferromagnetic coupling between the Type-3 copper atoms (Dwivedi et al., 2011).

Conclusion

On the basis of the results of sequence and phylogenetic analyses, expression profiling, and the 3-D structure modeling, the RPW laccase reported in this study belongs to the group of insect laccase 2. Tissue expression of *RfeLac2* highlights the release of this enzyme earlier than the onset of the RPW larval molting and then suppressed before the beginning of molting process. Further studies on targeting disruption of each functional motif of the *RfeLac2* gene are required for further clarification of the specific role of *RfeLac2* in the RPW during the growth and development.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary Table 1. List of laccases (Lacs) multicopper oxidase family enzymes (MCOs) from different insect species and their GenBank® accession numbers

Species	Order	Gene name	GenBank Accession #
Rhynchophorus ferrugineus	**Coleoptera**	Laccase 2 (*RfeLac2*)	MK655469
Tribolium castaneum		Laccase 1 (*TcaLac1*)	AAX84206.1
		Laccase 2 (*TcaLac2*)	NP_001034487.2
		Laccase 2A *(TcaLac2A)*	AAX84202.2
		Laccase 2B (*TcaLac2B*)	AAX84203.2
		Laccase 2 variant X1 (*TcaLac2X1*)	XP_008199220.1
Aethina tumida		Laccase 1-like (*AtuLac1*)	XP_019875844.1
Anoplophora glabripennis		Laccase (*AglLac*)	XP_018575474.1
Nicrophorus vespilloides		Laccase-like (*NveLac*)	XP_017781263.1
Leptinotarsa decemlineata		Laccase (*LdeLac*)	XP_023022290.1
Agrilus planipennis		Laccase 5 (*AplLac5*)	XP_018324480.1
Onthophagus taurus		Laccase (*OtaLac*)	XP_022900258.1
Monochamus alternatus		Laccase 2 (*MalLac2*)	ABU68466.1
Dendroctonus ponderosae		Laccase 5 (*DpoLac5*)	XP_019754547.1
Chrysomela populi		Laccase 2 (*CpoLac2*)	AWK23445.1
Phaedon cochleariae		Laccase 2 (*PcoLac2*)	AWK23446.1
Culex quinquefasciatus	**Diptera**	Multicopper oxidase 1 (*CquMCO1*)	XP_001867157.1
		Multicopper oxidase 1 variant 1 (*CquMCO1X1*)	XP_001861600.1
Culex pipiens pallens		Laccase 2 (*CppLac2*)	ACG63789.1
Aedes aegypti		Laccase 1 variant X1 (*AaeLac1X1*)	XP_021698133.1
		Laccase 4 isoform X4 (*AaeLac4X2*)	XP_021698134.1
		Multicopper oxidase 1 (*AaeMCO1*)	AAY29698.1
		Multicopper oxidase 2 (*AaeMCO2*)	AAY32604.1
Aedes albopictus		Laccase 4 isoform X2 (*AalLac4X2*)	XP_019553181.1
Anopheles sinensis strain LS-WX		Laccase 2 (*AsiLac2*)	ARG47519.1
Musca domestica		Laccase 2 (*MdoLac2*)	XP_005177649.2
Anopheles gambiae		Laccase 1 (*AgaLac1*)	AAN17505.1
		Laccase 2 isoform A (*AgaLac2A*)	AAX49501.1
		Laccase 2 isoform B (*AgaLac2B*)	AAX49502.1
		Laccase 3 (*AgaLac3*)	ABQ95972.2
		Multicopper oxidase 4 (*AgaMCO4*)	ABY84643.1
		Multicopper oxidase 5 (*AgaMCO5*)	ABY84644.1
Stomoxys calcitrans		Laccase 2 (*ScaLac2*)	XP_013106835.1
Lucilia cuprina		Laccase 2 (*LcuLac2*)	XP_023306400.1
Bactrocera (Zeugodacus) cucurbitae		Laccase 5 variant X1 (*BcuLac5X1*)	XP_011177989.1
Drosophila miranda		Laccase 5 (*DmiLac*5)	XP_017149067.1
Drosophila melanogaster		Multicopper oxidase 1 isoform A (DmeMCO1A)	NP_609287.3
		Laccase 2 (DmeLac2A)	NP_724412.1
		Multicopper oxidase 3 (DmeMCO3)	NP_651441.1
Manduca sexta	**Lepidoptera**	Laccase 1 (*MseLac1*)	AAN17506.1
		Laccase 2 (*MseLac2*)	AAN17507.1
Helicoverpa armigera		Laccase 1 (*HarLac1)*	XP_021185007.1
		Laccase 2 (*HarLac2*)	AHA15412.1
		Multicopper oxidase 1 (*HarMCO1*)	KP318028
Pieris rapae		Laccase 5 (*PraLac5*)	XP_022124207.1

Supplementary Table 1. Contd.

Antheraea pernyi		Laccase 2 (*ApeLac2*)	AII19522.1
Papilio machaon		Laccase 5 (*PmaLac5*)	NP_001303942.1
		Laccase 5 variant X1 (*PmaLac5X1*)	XP_014370419.1
Papilio polytes		Laccase 5 (*PpoLac5*)	NP_001298599.1
		Laccase 5 variant X1 (*PpoLac5X1*)	XP_013146294.1
Papilio xuthus		Laccase 5 (*PxuLac5*)	NP_001298899.1
		Laccase 5 variant X2 (*PxuLac5X2*)	XP_013180620.1
Spodoptera litura		Laccase 1 (*SliLac1*)	XP_022819652.1
Biston betularia		Laccase 2 (*BbeLac2*)	AEP43806.1
Bombyx mori		Multicopper 1 (*BmoMCO1*)	DAA06286.1
		Multicopper 2 isoform B (*BmoMCO2B*)	DAA06287.1
		Laccase (*BmoLac2*)	ABU68465.1
Acyrthosiphon pisum	**Hemiptera**	Laccase 1 (*ApiLac1*)	XP_003241886.1
		Laccase 5 (*ApiLac5*)	XP_001950788.1
Nephotettix cincticeps		Laccase 1 isoform G (*NciLac1G*)	BAJ06132.1
		Laccase 1 isoform S (*NciLac1S*)	BAJ06131.1
		Laccase 2 (*NciLac2*)	BAJ06133.1
Bemisia tabaci		Laccase 2 variant X2 (*BtaLac2X2*)	XP_018913180.1
		Laccase 2 variant X1 (*BtaLac2X1*)	XP_018913179.1
		Laccase 1 (*BtaLac1*)	AQY62684.1
Cimex lectularius		Laccase 5 (*CleLac5*)	XP_014240544.1
Nilaparvata lugens		Multicopper oxidase 2 (*NluMCO2*)	AKN21380.1
		Laccase (*NluLac5*)	XP_022184002.1
Riptortus pedestris		Laccase 2 (*RpeLac2*)	BAJ83487.1
Halyomorpha halys		Laccase 5 (*HhaLac5*)	XP_014271851.1
Diuraphis noxia		Laccase 5 (*DnoLac5*)	XP_015374008.1
Megacopta punctatissima		Laccase 2 (*MpuLac2*)	BAJ83488.1
Orussus abietinus	**Hymenoptera**	Laccase 5 (OabLac5)	XP_023290784.1
Cephus cinctus		Laccase-5-like (CciLac5)	XP_015602372.1
Fopius arisanus		Laccase 2 (FarLac2)	XP_011307332.1
Apis mellifera		Laccase 1 (AmeLac1)	XP_026295929.1
		Laccase 5 (AmeLac5)	XP_625189.3
		L- Ascorbate oxidase (AmeAsO)	XP_006562317.1
Diachasma alloeum		Laccase 5 (DalLac5)	XP_015111370.1
Neodiprion lecontei		Laccase 5 (NleLac5)	XP_015522336.1
Megachile rotundata		L-Ascorbate oxidase (MroAsO)	XP_012134606.1
Bombus impatiens		Laccase 1 variant X1 (BimLac1X1)	XP_003490974.1
Bombus terrestris		Laccase 1 (BteLac1)	XP_003399477.1
Harpegnathos saltator	**Hymenoptera**	Laccase 5 variant X1 (*HsaLac5X1*)	XP_011142481.1
		Laccase 5 variant X2 (*HsaLac5X2*)	XP_011142482.1
		Laccase 5 variant X3 (*HsaLac5X3*)	XP_011142483.1
Microplitis demolitor		Laccase 5 (*MdeLac5*)	XP_008557222.1
Cyphomyrmex costatus		Laccase 4 (*CcoLac4*)	XP_018394374.1
Trachymyrmex cornetzi		Laccase 4 (*TcoLac4*)	XP_018363669.1
Polistes canadensis		Laccase (*PcaLac*)	XP_014599609.1
Trachymyrmex zeteki		Laccase 4 (*TzeLac4*)	XP_018302362.1
Acromyrmex echinatior		Laccase 4 variant X1 (*AecLac4X1*)	XP_011062541.1
		Laccase 4 variant X2 (*AecLac4X2*)	XP_011062542.1
Ooceraea biroi		Laccase 5 (*ObiLac5*)	XP_011336181.1

Supplementary Table 1. Contd.

Trachymyrmex septentrionalis		Laccase 4 (*TseLac4*)	XP_018346813.1
Pseudomyrmex gracilis		Laccase (*PgrLac*)	XP_020298349.1
Nasonia vitripennis		Laccase 5 (*NviLac5*)	XP_016843007.1
Polistes dominula		Laccase (*PdoLac*)	XP_015186385.1
Camponotus floridanus		Laccase 5 (*CflLac5*)	XP_011259955.1
Linepithema humile		Laccase (*LhuLac5*)	XP_012216530.1
Pimpla hypochondriaca		Laccase (*PhyLac*)	CAD20461.1
Zootermopsis nevadensis	**Dictyoptera**	Laccase (*ZneLac*)	XP_021934069.1
Cryptotermes secundus		Laccase (*CseLac*)	XP_023707482.1
Gryllus bimaculatus	**Orthoptera**	Laccase 2 (*GbiLac2*)	BAM09185.1

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In vitro regeneration protocol through direct organogenesis for *Jatropha curcas* L. (Euphorbiaceae) accessions in Ethiopia

Hundessa Fufa[1*], Meseret Tesema[1] and Jiregna Daksa[2]

[1]School of Plant and Horticultural Sciences, College of Agriculture, Hawassa University; P. O. Box. 05, Hawassa, Ethiopia.
[2]Holeta Agricultural Research Center, Ethiopian Institute of Agricultural Research, Ethiopia.

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***Jatropha curcas* L. is among important tree crops in the world with a potential for biofuel production. In Ethiopia, there is a soaring investors' interest to produce Jatropha in the country for biodiesel production. However, insufficient good quality propagation material is a major production constraint. A study was undertaken to establish a protocol for *in vitro* mass propagation of three Ethiopian Jatropha accessions viz. Metema, Adami Tulu and Shewa Robit through direct organogenesis from nodal explant. The result revealed that the highest percentage of shoot induction (86-90%) was achieved on MS medium with BAP (1 mg/L) and IBA (0.5 mg/L) for all the three accessions. The maximum number of shoots (6) was obtained for Metema when BAP (0.5 mg/L) with Kn (0.5 mg/L) was used. Whereas, the maximum (3.2 cm) shoot length was recorded for Shewa Robit on media with 0.5 mg/L Kn. The highest rooting percentage (84.8-88%) and maximum root number (5.43) were recorded on media supplemented with 0.25 mg/L IBA. Shewa Robit and Metema had longer roots on media with 0.25 mg/L IBA. Finally, the plantlets were successfully established in greenhouse with survival rate of 86.67% for Shewa Robit followed by 73.33 and 66.67% for Metema and Adami Tulu, respectively. This study provided optimal protocol for micro-propagation of Jatropha accessions through direct organogenesis to boost its production.**

Key words: *Jatropha curcas*, biofuel, organogenesis, plant growth regulators.

INTRODUCTION

Jatropha (*Jatropha curcas* L.) is a succulent shrub or small tree, which belongs to the large Euphorbiaceae family. It originated from Central America. From the Caribbean, Jatropha was probably distributed by Portuguese seafarers via the Cape Verde Islands and former Portuguese Guinea (now Guinea Bissau) to other countries in Africa and Asia in the 16th century (Heller, 1996; BAZ, 2007). It is currently found worldwide in most tropical countries including Ethiopia (Mekuria et al., 2008).

*Corresponding author. E-mail: hundesa9074@gmail.com.

Jatropha is a multi-purpose plant which has been exploited for various purposes such as soil erosion control, firewood, hedges, green manure and traditional medicines (Carels, 2013). On the other hand, the seed oil of Jatropha is also used as soap manufacturing ingredient, paints and as a biodiesel to substitute kerosene (Kumar and Sharma, 2008). Among many other attributes and importance of Jatropha, in recent years it has gotten special attention for being a priority feed stock in production of biodiesel.

Biodiesel is an alternative diesel fuel made from different types of renewable sources such as plant oils and animal fats. It is environmentally friendly fuel with low emission profiles and also non-toxic and biodegradable (Abdulla et al., 2011). Biofuels derived from non-edible oils such as Jatropha (*J. curcas*), Mahua (*Madhuka indica*), Cardoon (*Cynara cardunculus*), Paradise tree (*Simarouba glauca*), Castor (*Ricinus communis*), Karanj (*Pongamia pinnata*) are more economical and suitable compared to edible oils (Naresh et al., 2012). In some of the developed nations (US and European countries) edible oils such as rape seed and soybean oils are used for biodiesel conversion. However, in developing countries usage of edible oils for biofuels is not feasible (Knothe, 2000).

Among the plant species producing raw materials for biofuels, Jatropha is one of the plant species that stimulates the highest interest in tropical and subtropical regions. It has been identified as most suitable oil seed bearing plant due to its various favorable attributes like high oil content, hardy nature, adaptability in a wide range agro-climatic conditions, need for less irrigation and less agricultural inputs, pest resistance, short gestation periods and suitable traits for easy harvesting (Heller, 1996; Edrisi et al., 2015).

In Ethiopia, Jatropha grows in various parts of the country such as in Wolayita, Metekel, Southern Wollo, Northern and Eastern Shoa, Tigray, Gamo Gofa zones and Gambella region (Getinet et al., 2009). Traditionally, farmers in Ethiopia mainly used Jatropha plants as living fences and as a structural means of conserving soil and water (Zufan, 2010). Now, in connection with green economy goals, the Ethiopian government has begun to promote supply of fuels from locally produced biofuel without affecting food self-sufficiency and by reducing environmental impacts (FDRE, 2007, 2011). The strategy intends to make the country carbon neutral by 2030 (FDRE, 2011). To achieve these goals, biofuels which can be produced from non-edible oil like Jatropha is the best solution.

Based on this strategy several local and foreign private investors have started growing plants for producing biodiesel. There are about 85 companies registered in Ethiopia to invest in biofuels, mainly Jatropha (Mengistu, 2013). Most of these companies have the intention of going for large-scale commercial development (Abreham and Belay, 2015). However, several challenges remain before the plant biomass can be commercially exploited. Its supply on a large scale requires massive production of phenotypically uniform plant material of a very high quality within a short time-frame that is adapted to the growth conditions of the plantation areas. The increase in plantation area creates high demand of good planting material to be available and these calls for a means that can provide planting material in large scale and within short period of time. There is a need to establish mass multiplication technique to meet the large-scale demand and easy supply of Jatropha plant (Medza Mve1 et al., 2013; Mengistu, 2013).

Traditionally Jatropha is propagated through seed and vegetative cutting. The most common method to obtain Jatropha plantlets is by seed germination, which can be severely limited by poor seed viability, low germination percentage, inadequate rooting in growth plants in small pots and the delayed rooting of seedlings (Openshaw, 2000). Seed propagated plants are also not true to type and can result in oil concentration variations between 8-54% (Ovando-Medina et al., 2011).

Vegetative propagation of Jatropha through stem cuttings has been achieved however the established plants are not deep rooted and hence, they easily get uprooted when cultivated in lands with poor top soil (Openshaw, 2000). Despite their profuse vegetative growth, the number of seeds produced per plant is very low and the seeds show a low seed fecundity, which is reduced by 50% within 15 months (Ginwal et al., 2004). Plants propagated by cuttings also show a lower longevity and possess a lower drought and disease resistance than those propagated by seeds (Sujatha et al., 2005). Again this conventional propagation technique has its own draw back since planting materials are one of sources for disease transmission from place to place (Genene, 2014). Besides, for an effective large scale commercialized production of Jatropha maintaining true to type genotypes, producing disease free planting materials and high number of propagule *in vitro* culture has a paramount importance. Therefore, to improve cultivation of this crop, the traditional inefficient mode of propagation should be changed and proper techniques need to be studied and put in place for mass production of the Jatropha plants.

The *in vitro* multiplication would be a useful alternative method for mass production of the plant. Micropropagation technique (Plant tissue culture) offers an opportunity for large scale production of uniform disease free planting material in a relatively short period of time and independent of the season (George, 2008). *In vitro* derived plants are frequently more vigorous and of superior quality compared to those produced by *in vivo* methods. Evaluation of tissue culture propagated plants of Jatropha revealed that they produced a better yield and yield- related traits than seed-propagated plants (Sujatha et al., 2005). That means this clonal propagation method has the advantage of producing plants that are

Table 1. Sources and growing altitudes of planting material of Jatropha accessions were used in this study.

Province (Region)	Place of collection	Altitude(m)	Collectors (seed source)
Oromia	Adami Tulu	1500	WGRC
Amhara	Metema	1000	SARC
Amhara	Shewa Robit	1250	SARC

WGRC= Wondo Genet Research Center; SARC= Sirinka Agricultural Research Center.

morphologically homogenous with an equal production potential. The culturing of plant cells or organs can overcome problems of the flowering season, pollination, pollinators, seed setting, gestation period, viral infections, etc. Besides, Jatropha is a perennial crop where flowering would mostly take more than a year in majorities of the genotypes. This would in turn hinder getting sufficient amount of seed for rapid propagation. Therefore the development of an efficient *in vitro* regeneration system would be a remarkable progress for the Jatropha business and the field of alternative energy technology (Rajore and Batra, 2007).

One of the prospective and potential ways of *in vitro* plant culture of Jatropha is organogenesis. Organogenesis refers to the process in which a unipolar structure can be derived either through differentiation of non-meristematic tissues or through pre-existing meristematic tissues (Thorpe et al., 1990; Hussain et al., 2012; Moniruzzaman et al., 2016). It is one of the widely used methods employed for *in vitro* plant regeneration. Plant regeneration through organogenesis can occur either directly or indirectly (Thorpe et al., 1990; Geetha et al., 2008).

Direct organogenesis involves the emergence of adventitious organs directly from the meristematic region of the explants without callus phase and important to ensure clonal fidelity. Several authors have regenerated Jatropha through organogenesis using different explants (Sujatha and Muktra, 1996; Sujatha et al., 2005; Rajore and Batra, 2005; Deore and Johnson, 2008; Kumar et al., 2011).

Plant regeneration from *in vitro* organogenesis is possible due to the induction of stimuli for certain metabolic pathways that will trigger changes in the pattern of cell growth and development (Almeida et al., 2012). The regulation of organogenesis *in vitro* can be achieved by different types of manipulation. These include appropriate choice of explant, age of the explant, orientation of explant, proper choice of the culture medium, plant growth regulators, genotype, source of carbohydrate, gelling agent and other physical factors including light regime, temperature and humidity (Sujatha and Mukta, 1996; Sujatha et al., 2005; Feyissa et al., 2005; Deore and Johnson, 2008).

Among factors influencing *in vitro* regeneration via organogenesis, plant growth regulators in media and genotypes are the most important. The plant growth regulators (e.g., auxins and cytokinins) play an important role in organogenesis processes since their regimes can be used to manipulate the morphogenetic response of plants under in vitro cultures (Arya et al., 2009). *In vitro* plant regeneration from explants requires the presence of appropriate concentrations and combinations of plant growth regulators in the culture media (Kalimuthu et al., 2007). A sub-optimal culture medium may cause physiological disorders or death of tissue. Studies of auxins and cytokinins separately or their combinations to initiate *in vitro* organogenesis in Jatropha were reported (Sujatha et al., 2005; Deore and Johnson, 2008). In addition, genotypic differences in shoot organogenesis have been observed in a wide range of species including Jatropha. It has been reported that regeneration in Jatropha is highly genotype dependent (Kumar, 2008; Kumar and Reddy, 2010; Kumar et al., 2010; Mweu et al., 2016).

In Ethiopia, development and application of tissue culture techniques for propagation of Jatropha is at its early stage. Prior to this work, there are no enough documented studies on micropropagation of Ethiopian Jatropha accessions which can be used for mass production. Keeping in view of the importance of the crop and its propagation methods, the present study was designed to optimize *in vitro* protocol for direct organogenesis of three Ethiopian Jatropha accessions using nodal explant. Hence, the specific objectives of the study were:

(i) To optimize the concentrations and combinations of different growth regulators on MS medium for maximum proliferation of shoots from direct organogenesis;
(ii) To investigate rooting response of shoots to different IBA and NAA concentrations and combinations;
(iii) To evaluate survival rates of acclimatized plant regenerants in green house environment.

MATERIALS AND METHODS

Planting material

The seed of three Jatropha accessions were collected from Amhara and Oromia region of Ethiopia and used for these tissue culture experiments (Table 1).The seeds were germinated on growth trays containing sterilized combination of soil, sand and manure in the ratio of 2:1:1, respectively and kept in the greenhouse condition of Holeta Agricultural Research Center (HARC). They were watered thrice a week using a spraying can. After three weeks, the seed that germinated was transplanted into pot containing sterilized soil and

Figure 1. Mother stock plant of Jatropha accessions after three months.

kept as mother stock plant. After three months of growth (Figure 1); very young, health and vigorous part of the plant (nodal segment) was collected and used as a source of explants. The overall experiment (June 29, 2017 to January 30, 2018) was conducted at Plant Biotechnology Laboratory of Holeta Agricultural Research Center (HARC) 45 km West of Addis Ababa, Ethiopia.

Growth regulators stock preparation

The Plant Growth regulators (PGRs) used for the study were the cytokinin, 6-benzyl aminopurine (BAP) and Kinetin (Kn), and the auxins, indole-3- butyric acid (IBA) and α-naphthalene acetic acid(NAA). All PGRs stock solutions were prepared by weighing and dissolving the powder in distilled water at the ratio of 1 mg/ml. To begin the dissolving process, the powdered crystal of the PGRs was first weighed and dissolved in 3-4 drops of 1N NaOH and 1N HCl based on the type of PGR (NaOH for auxins and HCl for cytokinin). Then, the volume was adjusted by adding distilled water. Finally, growth regulators' stock solutions were stored in a refrigerator at +4°C for short term use.

Culture medium preparation

Culture medium was prepared by taking the proper amount of Murashige and Skoog (1962) stock solutions (mg/L). Full-strength of MS with 30 g/L of sucrose as carbohydrate source (w/v) for shoot initiation and multiplication were used whereas, half-strength of MS with 15 g/L sucrose were used for root induction. The pH of the medium was adjusted to 5.8 (using 1N NaOH and 1N HCl) after addition of the growth regulators. Gerlite (2.5 g/L) was added as a gelling agent after the volume and pH of the medium was adjusted. The media was sterilized by autoclaving at a temperature of 121°C with a pressure of 15PSi for 15 min and stored at room temperature.

Explants and surface disinfection

Nodal explant was collected from healthy and vigorously growing mother stock plants. The excised explant materials were initially rinsed under tap water for 30 min to remove the dust particles from their surface. Then the explants were treated with commercial detergent (Largo, Ethiopia) for 5 min and were rinsed well with distilled water for three to five times. Under a clean Laminar flow hood, the explants were subjected to 70% (v/v) ethanol for one minute and rinsed with sterile distilled water three to four times. Further the explant materials were then surface sterilized by 3% of Local bleach (Berekina, Ethiopia) containing two drops of Tween 20 for 15 min time of exposure. After that the explants were rinsed with sterilized double distilled water for three to four times to remove the residual effect of these sterilants. After sterilization process was completed, individual nodal segment was trimmed (l -l.5 cm) with one node and inoculated by vertical orientation on the medium.

Culture initiation

For culture initiation, single nodal explant (1-1.5 cm) was inoculated on full strength MS media supplemented with different combinations of BAP (0, 1, 1.5 and 2.0 mg/L) and IBA (0, 0.5 and 1.0 mg/L) along with Ascorbic acid (10 mg/L) for prevention of browning of cultures. Five nodal explants per culture jars and five replications for each treatment were used. The cultured explants were incubated in the light condition (1500 - 2000 lux) in growth room at 25 ± 2°C for four weeks.

Shoot multiplication and elongation

For shoot multiplication, shoots from best establishment (induction) medium were used to avoid the influence of different origin of media. Then, highly aseptically initiated shoots were transferred to shoot multiplication MS fresh medium which supplemented with various combinations of BAP (0, 0.5, 1.0, 1.5 mg/L) and Kn (0, 0.5 and 1.0 mg/L). Five shoots per culture jars and five replications for each treatment were used. The cultures were maintained at 25 ± 2°C with a 16 h photoperiod at a light intensity of 1500 - 2000 lux from cool white florescent bulbs. After four weeks of culture, the growth response of the micro-shoots to different treatments was recorded. For shoot elongation, the multiplied shoots were transferred onto growth regulators free MS basal medium for two weeks.

Root induction

The elongated shoots with three to four leaves were excised and cultured on half strength MS media supplemented with different

Table 2. Percentage of shoot initiation from nodal explant cultures of three Jatropha accession (Metema, Adami Tulu and Shewa Robit) at different concentrations and combinations of BAP and IBA after 30 days of culture.

PGRs (mg/L)		Shoot induction (%)		
BAP	**IBA**	**Metema**	**Adami Tulu**	**Shewa Robit**
0	0	-	-	-
0	0.5	34.80 ±0.20^{op}	35.20 ±0.0.20^{o}	33.00 ±1.22^{p}
0	1.0	-	-	-
1.0	0	70.00 ±0.00^{g}	68.00 ±1.22^{h}	69.80 ±0.20^{gh}
1.0	0.5	90.00 ±0.31^{a}	86.00 ±1.00^{b}	90.00 ±0.00^{a}
1.0	1.0	24.00 ±0.00^{q}	23.14 ±0.00^{q}	25.00 ±0.5^{q}
1.5	0	74.00 ±1.00^{ef}	73.00 ±1.22^{f}	75.00 ±0.00^{e}
1.5	0.5	80.00 ±0.00^{d}	80.00 ±0.00^{d}	82.00 ±1.22^{c}
1.5	1.0	55.00 ±0.00^{k}	52.00 ±1.22^{l}	53.00 ±1.22^{l}
2.0	0	85.00 ±0.00^{b}	85.00 ±0.00^{b}	85.00 ±0.00^{b}
2.0	0.5	63.00 ±1.22^{j}	65.00 ±0.00^{i}	64.00 ±1.00^{ij}
2.0	1.0	41.00 ±1.00^{n}	43.00 ±1.22^{m}	40.00 ±0.00^{n}
CV (%)			**2.92**	
LSD (5%)			**1.86**	

Different letters (within columns and rows) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=5). CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard error, n= Number of samples, '-' indicates no response.

combinations of IBA (0, 0.25, 0.5 and 1.0 mg/L) and NAA (0, 0.25 and 0.5 mg/L) for root induction. There were five replicates with five shoots cultured for each jars. Then the culture was maintained in a growth room at a temperature of 25±2°C and 16 h photoperiod provided by white florescent lamps. Each root growth parameters were recorded within four weeks of the culture.

Growth conditions

All the cultures were kept under eight hour dark period and sixteen hour photoperiod in a growth room. Artificial light was provided by parallel cool white fluorescent tubes (Philips, India) installed above the cultures. The light intensity was regulated to 1500-2000 lux and the growth room temperature was adjusted at 25 ± 2°C with relative humidity (RH) of 65-70%.

Acclimatization

After the well rooted *in vitro* propagated Jatropha plantlets are obtained, it was taken out gently from the culture vessels and the root system was washed under running tap water to remove traces of agar that prevent the absorption of nutrients from the acclimatization culture substrates by roots. A total of 45 well rooted shootlets (15 shootlets from each Jatropha accession) were transferred to plastic pots containing a mixture of sterilized sand, soil and compost in the ratio of 1:2:1, respectively and then transferred to the greenhouse for hardening. The potted plants were maintained in a greenhouse at a temperature of 25 ± 2°C with 60-75% relative humidity. The pots were covered with transparent plastic bags with random holes for air circulation and the underside of the pots was drilled for drainage. Then they were watered using sprayer every day. Plastic cover were removed partially after a week and completely removed after two weeks. Finally, after about one month, percent of plantlets successfully hardened were calculated.

Experimental design and statistical analysis

The experiment was laid out in Completely Randomized Design (CRD) for all the treatments. The experiment was comprised of different combination and concentrations of plant growth regulators combined with three accessions of Jatropha. Growth regulators were one factor and accessions were another factor. Each treatment had five replicates of culture Jars and set as experimental unit. Data collected from each experiment was subjected to statistical analyses using the SAS statistical software (version 9.2) and ANOVA was constructed, followed by mean separation using Fisher's Least Significance Difference (LSD) at α=5%.

RESULTS AND DISCUSSION

Effects of BAP and IBA on Shoot Induction

Analysis of variance showed that, there was highly significant difference (P<0.01) among growth regulator concentrations and combinations (BAP and IBA) and their interaction with the three Jatropha accession on percentage of shoot initiation. ANOVA also revealed that there was no significant difference (P>0.05) among the Jatropha accessions on percentage of shoot induction (Table 2).

The highest percentage (90%) shoot induction was recorded for both Shewa Robit and Metema accessions on MS media supplemented with combination of 1 mg/L BAP and 0.5 mg/L IBA followed by 86% for Adami Tulu accession on the same hormone combinations and concentrations. Whereas, the lowest percentage (22-25%) induction was observed from the media containing

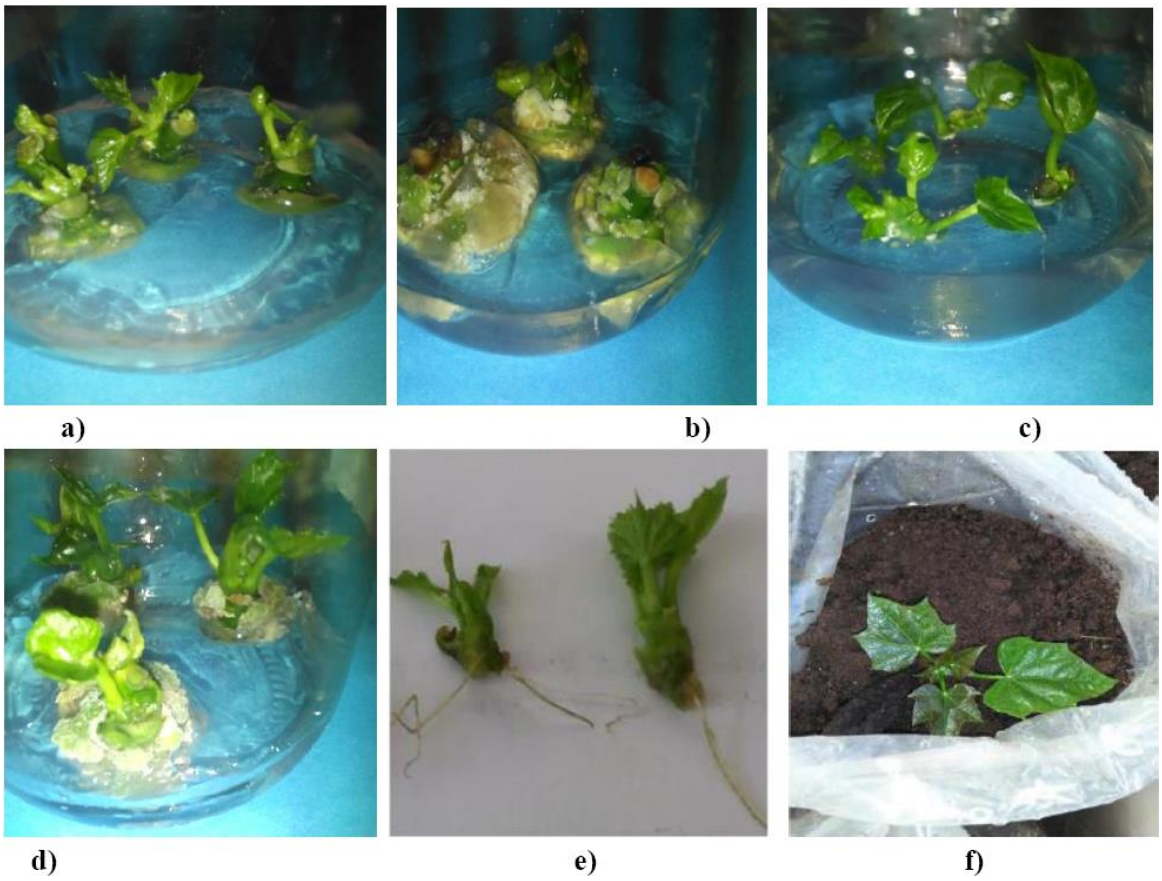

Figure 2. Plant regeneration in Jatropha through direct organogenesis. (a) Shoot induction on 1.5 mg/L of BAP from nodal explant, (b) MS medium containing 1 mg/L of IBA, (c) Shoot multiplication on 0.5 mg/L BAP, (d) Callus formation on rooting MS media with 0.5 mg/L NAA, (e) Rooting on half- strength medium with 0.25 mg/L IBA, (f) plantlet undergoing acclimatization.

combination of 1 mg/L of BAP and 1 mg/L IBA for all accessions (Table 2). Addition of IBA along with BAP has also been reported to regenerate shoot buds from the nodal explants in Jatropha (Shrivastava and Banerjee, 2008). This is mainly due to the reason that, the hormone balance is apparently more important than the absolute concentration of any one hormone since plant hormones do not function in isolation within the plant body, but, instead, function in relation to each other (Deore and Johnson, 2008). Both cell division and cell expansion occur in actively dividing tissue, therefore cytokinin and auxin balance plays a role in the overall growth of plant tissue (Jha et al., 2007; Shrivastava and Banerjee, 2008; Purkayastha et al., 2010).

However, in this study good results were also obtained on MS medium which contains only BAP (2 mg/L). This could be due to the endogenous auxin which balances with cytokinin releasing the shoot buds from apical dominance. Several studies have reported that BAP is the most suitable cytokinin for shoot induction and multiplication in Jatropha. Maharana et al. (2012) have also reported that MS medium fortified with 8.0 µ*M* BAP regenerated shoot buds (6.2 shoots) from nodal explants of Jatropha. On the other hand, *in vitro* growing shoots of Jatropha had revealed a tendency of callusing when cultured on a medium fortified with sole IBA, at a concentration of 1 mg/L (Figure 2b). Hence we can say that an optimum ratio of cytokinin to auxin is essential for proper shoot bud formation. The study by Xiansong (2010) indicated that the sole effect of BAP, IBA and their interactions had significant effects on plant regeneration and type of regeneration in sweet potato. This is supported by many researches implying that the interaction between auxin and cytokinin is important for the regulation and guiding developmental processes, such as the formation and maintenance of the meristem and formation of callus which are crucial mechanisms for the establishment explant (Kalimuthu et al., 2007).

Effects of BAP and kinetin on shoot multiplication

The result showed that number of leaves per shoot was highly significantly ($P<0.01$) affected due to the main

Table 3. Main effect of the different concentrations and combinations of Cytokinins (BAP and Kn) on number of leaves per shoot of Jatropha accessions.

PGR concentration (mg/L)		Number of leaf per shoot
BAP	Kinetin(Kn)	
0	0	3.43±0.08^{hi}
0	0.5	4.00±0.05^{g}
0	1.0	4.50±0.09^{f}
0.5	0	7.00±0.09^{a}
0.5	0.5	6.43±0.09^{b}
0.5	1.0	5.00±0.05^{e}
1.0	0	5.53±0.10^{d}
1.0	0.5	6.00±0.17^{c}
1.0	1.0	4.13±0.06^{g}
1.5	0	2.97±0.03^{j}
1.5	0.5	3.67±0.11^{h}
1.5	1.0	3.30±0.05^{i}
LSD (5%)		0.27
CV (%)		7.93

Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=15). CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard Error, n=number of samples.

effect of PGRs concentrations and combinations, however, the effect of accessions and interaction of the two factors affected the number of leaves non-significantly (P>0.05). ANOVA also showed that genotype (Jatropha accessions), PGRs and their interactions had very high significant (P<0.01) effects on average shoot length and average shoots number (Table 3).

The maximum mean number of leaf (7 per shoot) was recorded on MS media supplemented with 0.5 mg/L of BAP followed by (6.43 per shoot) on media supplemented with combination of 0.5 mg/L of BAP and 0.5 mg/L of Kn. Whereas, the lowest mean number of leaf (2.97 per shoot) was recorded for shoots induced on MS media supplemented with 1.5 mg/L of BAP (Table 3). In this study a significant influence of the type of cytokinins and their concentrations on the number of leaves per shoot was observed. As the concentration of Kn increased the number of leaf per shoot also increased. However, the cultures growing on the media containing higher BAP produced the smallest number of leaves. Kozak and Salata (2011) reported that the lower concentration of BAP (0.5 mg/L) produced maximum number of leaves (16.8 leaves) than other concentration of Kinetin, 2iP and TDZ in *Rheum rhaponticum*. Similarly, Behera et al. (2014) obtained highest number of leaf per shoot (7.1) on MS medium supplemented with 1 mg/L BAP and 1 mg/L IAA in Jatropha. More recently Rady et al. (2016) also reported that MS supplemented with combination of 0.5 mg/l BA and 0.05 mg/L IBA gave the highest number (14.67) of leaves per proliferated shoot bud of Jatropha after one month of cultivation in the growth room.

The maximum number of shoots was recorded for Metema (6 shoots) on MS media supplemented with combination of 0.5 mg/L BAP and 0.5 mg/L Kn followed by 5.70 and 5.56 shoots for Shewa Robit and Adami Tulu accessions, respectively, on the same growth regulator combination and concentration as for Metema. Whereas, the lowest mean number (1.88-1.96) of shoots of the experiment was recorded for all the three accession shoots on growth regulator free MS media (Table 4). In this study, the optimum combination of BAP and Kn which was 0.5 mg/L BAP and 0.5 mg/L Kn showed better response on number of shoot per explants for all accession than the other treatments. This might be due to cytokinins participate in the regulation of cell division which leads to shoot bud formation from the explant. This is in line with the earlier studies where in media containing kinetin in conjunction with BAP induced higher frequency of shoot multiplication and greater number of shoots in some perennial plants (Figueiredo et al., 2001; Baskaran and Jayabalan, 2005). Weaker effect on axillary shoot regeneration for all the three accessions was observed in hormone free treatments. Thus results confirmed with Thepsamran et al. (2008) who reported that exogenous application of cytokinins has become obligatory for induction of multiple shoot in Jatropha.

In case of shoot length, the maximum shoot length was recorded for Shewa Robit (3.2 cm) on media supplemented with 0.5 of mg/L Kinetin. Whereas, the lowest mean shoot length (1.8-1.84 cm) was recorded for all the three accession shoots developed on media

Table 4. Effect of different concentration and combination of BAP and Kn on number of shoots and shoot length of three Jatropha accessions.

Jatropha Accessions	Cytokinin (mg/L)		No. of shoot per explant	Length of shoot (cm)
	BAP	Kn		
Metema	0	0	1.96 ±0.04^{r}	3.00±0.00 ^{b}
	0	0.5	2.10 ±0.03^{pqr}	3.04 ±0.02^{b}
	0	1.0	3.00 ±0.00^{lm}	2.90 ±0.00^{c}
	0.5	0	4.60 ±0.10^{de}	2.48 ±0.02^{i}
	0.5	0.5	6.00 ±0.31^{a}	2.80 ±0.03^{e}
	0.5	1.0	5.50 ±0.16^{b}	2.60 ±0.00^{f}
	1.0	0	4.10 ±0.10^{fg}	2.18 ±0.02^{kl}
	1.0	0.5	3.72 ±0.09^{hi}	2.34 ±0.02^{j}
	1.0	1.0	3.40 ±0.03^{jk}	2.06 ±0.02^{mn}
	1.5	0	3.50 ±0.00^{ijk}	1.80 ±0.00^{q}
	1.5	0.5	2.78 ±0.02^{mn}	2.10 ±0.03^{m}
	1.5	1.0	2.40 ±0.00^{op}	1.92 ±0.05^{p}
Adami Tulu	0	0	1.88 ±0.05^{r}	3.00 ±0.00^{b}
	0	0.5	2.06 ±0.06^{qr}	3.02 ±0.02^{b}
	0	1.0	2.84 ±0.10^{m}	2.87 ±0.02^{cd}
	0.5	0	4.00 ±0.00^{fgh}	2.50 ±0.00^{hi}
	0.5	0.5	5.56 ±0.23^{b}	2.82 ±0.02^{de}
	0.5	1.0	4.70 ±0.20^{cd}	2.56 ±0.02^{fgh}
	1.0	0	3.80 ±0.12^{ghi}	2.20 ±0.03^{k}
	1.0	0.5	3.68 ±0.07^{ij}	2.34 ±0.02^{j}
	1.0	1.0	3.33 ±0.00^{k}	2.10 ±0.00^{m}
	1.5	0	3.30 ±0.12^{kl}	1.80 ±0.00^{q}
	1.5	0.5	2.80 ±0.00^{mn}	2.06 ±0.02^{mn}
	1.5	1.0	2.34 ±0.04^{opq}	1.96 ±0.04^{op}
Shewa Robit	0	0	1.92 ±0.05^{r}	3.00 ±0.00^{b}
	0	0.5	2.02 ±0.08^{r}	3.20 ±0.00^{a}
	0	1.0	2.96 ±0.04^{m}	2.92 ±0.02^{c}
	0.5	0	4.30 ±0.12^{ef}	2.52 ±0.02^{ghi}
	0.5	0.5	5.70 ±0.12^{ab}	2.80 ±0.00^{e}
	0.5	1.0	5.00 ±0.00^{c}	2.58 ±0.02^{fg}
	1.0	0	4.00 ±0.16^{fgh}	2.22 ±0.02^{k}
	1.0	0.5	3.80 ±0.00^{ghi}	2.36 ±0.04^{j}
	1.0	1.0	3.53 ±0.12^{ijk}	2.12 ±0.02^{lm}
	1.5	0	3.70 ±0.12^{hij}	1.84 ±0.04^{q}
	1.5	0.5	2.84 ±0.04^{m}	2.10 ±0.00 ^{m}
	1.5	1.0	2.50 ±0.16^{no}	2.00 ±0.00^{no}
	CV (%)		6.98	**2.03**
	LSD (5%)		0.30	**0.06**

Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=5). CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard Error.

supplemented with 1.5 mg/L BAP (Table 4). On the other hand, the highest mean shoot length for Metema and Adami Tulu accession were obtained from both MS medium containing 0.5 mg/L Kn and PGRs free MS medium with no significant difference to the accessions type as well as hormone concentrations from which the shoot buds were raised. The increased response of shoot length to PGRs free media could partly relate to the

endogenous levels of hormone in explants. On the other hand, the impact of Kn on shoot length of all the three accession showed better response than the other treatments. This result also confirmed with Jeevan et al. (2013) who reported that 1.0 mg/L kinetin gave the highest shoot length than media which containing BAP alone during *in vitro* culture of Jatropha. This shows that Kn is effective in driving shoot elongation and this is might be due to the reason that Kn is easy and readily to be absorbed by plant cells as compared to BAP. Kaminek (1992) also reported that variation in the activity of different cytokinins can be explained by their different uptake rate in different genomes, translocation rates to meristematic regions and metabolic processes in which cytokinin may be degraded or conjugated with sugars or amino acids to form biologically inert compounds.

Effects of IBA and NAA on rooting induction

ANOVA showed very highly significant ($P<0.01$) effect of all main and interaction effect of accession, IBA and NAA on rooting percentage and root length of *in vitro* rootinduction of the three Jatropha accessions. The result showed also that number of roots induced per shoot was highly significantly ($P<0.01$) affected due to the main effect of PGRs concentrations and combinations, however, the effect of accessions and interaction of the two factors affected the number of roots non-significantly ($P>0.05$).

The highest rooting percentage (88%) was recorded for Shewa Robit accession on media supplemented with 0.25 mg/L IBA followed by 86 and 84.8% for Metema and Adami Tulu accessions, respectively, on the same IBA concentration as for Shewa Robit. Whereas, the lowest rooting percentage (38.6-40.2%) were recorded for all accession shoots induced on media supplemented with combination of 1 mg/L IBA and 0.5 mg/L NAA (Table 5). On other hand, there is no root formed on the media supplemented with NAA alone. On this medium NAA often induces the formation of callus at the base of the shoot and rooting response was almost negligible (Figure 2d). Similar results to the present study were obtained by Shrivastava and Banerjee (2008) and Maharana et al. (2012). These authors observed that well-developed shoots of Jatropha when transferred to half MS medium fortified with NAA intermittent callus formation takes place and no roots were observed. The reason for low performance of NAA treatments may be due to the reason that NAA is more persistent than IBA, remains present in the tissue and may block further development of root meristemoids (Nanda et al., 2004). Studies also showed that, NAA was more consistent in stimulating cell divisions which favors callus formation (Kim et al., 2003). The promotory effect of IBA on *in vitro* rooting of shoots has also reported in Jatropha (Rajore and Batra, 2005; Kochhar et al., 2005) and they concluded that IBA alone was effective in the rooting of Jatropha.

The maximum mean root length was obtained for Shewa Robit (4.3 cm) on the media supplemented with 0.25 mg/L IBA followed by 4 and 3.8 cm for Metema and Adami Tulu accession, respectively on the same hormone concentration as for Shewa Robit. Meanwhile, the lowest root length (1.88-1.92) of the experiment was recorded for all the three accession shoots developed on hormone free half MS media (Table 5). In this study, the optimum level of IBA concentration is 0.25 mg/L for all the three accessions. Datta et al. (2007) have also reported that half MS medium fortified with 0.2 mg/L regenerated better root length (8.7 cm) from nodal explants of Jatropha. The results also revealed that root length tend to reduce with higher than optimum concentration of IBA. Kollmeier et al. (2000) reported that root elongation phase is very sensitive to auxin concentration and it is inhibited by high concentration of auxin in the rooting medium. It is possible that supra-optimal concentration of auxins inhibit root elongation through enhancement of ethylene biosynthesis which is root growth inhibitor (Hartman et al., 2009).

In case of root number the maximum root number (5.43) was recorded on MS media supplemented with 0.25 mg/L of IBA followed by (4.8) on media supplemented with combination of 0.5 mg/L of IBA and 0.25 mg/L of NAA (Table 6). Whereas, the lowest mean number (1.8) of roots per shoot was recorded for roots induced on PGR free MS media. On other hand, there is no root formed on the media supplemented with NAA alone due to callus formation at the base of shoot. In this study the root number decreased with higher concentration of IBA. These results are in line with Datta et al. (2007), who reported 0.2 mg/L IBA for best rooting in Jatropha. However, there are other studies who recommended 3 mg/l IBA addition to half MS medium for best rooting in Jatropha (Shrivastava and Banerjee, 2008). This deviation is owed the variation to the underlining genetic differences of the genotypes in response to rooting media composition and affecting rooting and other associated developments.

Besides, the decline in root number beyond the optimum level in this study might be due to the toxic effect of IBA beyond certain level which affects root growth and development. This observation is in agreement with the report of Thomas (2007) in *Curculigo orchioides* where a higher level of IBA produced a negative effect resulting in lower root number. Moreover, high levels of IBA can result in ethylene accumulation in the tissue culture vessel, which also inhibits the induction of root primordia (De Klerk, 2002; Hartman et al., 2009).

Acclimatization

The acclimatization results revealed that the highest percentage of survival rate of shoots (86.67%) was recorded for Shewa Robit plantlets whereas 73.33 and 66.67% recorded for Adami Tulu and Metema

Table 5. Effects of different concentration and combination of IBA and NAA on rooting percentage and root length of three Jatropha accessions.

Jatropha accession	Auxin (mg/L)		Rooting response (%)	Root length (cm)
	IBA	NAA		
Metema	0	0	51.20 ±0.66^{mn}	1.92 ±0.05^{n}
	0	0.25	-	-
	0	0.5	-	-
	0.25	0	86.00 ±0.63^{b}	4.00 ±0.00^{b}
	0.25	0.25	76.200 ±0.20^{f}	2.74 ±0.10^{gh}
	0.25	0.5	64.00 ±0.55^{ij}	2.58 ±0.05^{hi}
	0.5	0	78.80 ±0.37^{e}	3.20 ±0.12^{e}
	0.5	0.25	84.20±0.20^{cd}	3.62 ±0.07^{cd}
	0.5	0.5	59.20 ±0.58^{k}	2.30 ±0.12^{jkl}
	1.0	0	71.60 ±0.51^{h}	3.00 ±0.00^{ef}
	1.0	0.25	54.40 ±0.68^{l}	2.26 ±0.06^{jklm}
	1.0	0.5	40.20 ±0.49^{o}	2.08 ±0.02^{lmn}
Adami Tulu	0	0	50.40 ±0.60^{n}	1.88 ±0.05^{n}
	0	0.25	-	-
	0	0.5	-	-
	0.25	0	84.80 ±0.86^{bc}	3.80 ±0.00^{bc}
	0.25	0.25	74.80 ±0.73^{g}	2.87 ±0.03^{fg}
	0.25	0.5	63.60 ±0.24^{j}	2.48 ±0.13^{ij}
	0.5	0	80.00 ±0.00^{e}	3.50 ±0.16^{d}
	0.5	0.25	83.20 ±0.37^{d}	3.60 ±0.24^{cd}
	0.5	0.5	59.40 ±0.40^{k}	2.36 ±0.10^{ijk}
	1.0	0	72.00 ± 0.00^{h}	2.80 ±0.12^{fgh}
	1.0	0.25	55.00 ±0.45^{l}	2.22 ±0.09^{klm}
	1.0	0.5	38.60 ±0.81^{p}	2.06 ±0.02^{mn}
Shewa Robit	0	0	52.20 ±0.20^{m}	1.88 ±0.05^{n}
	0	0.25	-	-
	0	0.5	-	-
	0.25	0	88.00 ±0.00^{a}	4.30 ±0.00^{a}
	0.25	0.25	76.00 ±0.32^{fg}	2.79 ±0.09^{fgh}
	0.25	0.5	65.00 ±0.45^{i}	2.74 ±0.02^{gh}
	0.5	0	76.60 ±0.98^{f}	3.60 ±0.10^{cd}
	0.5	0.25	83.60 ±0.68^{cd}	3.68 ±0.07^{cd}
	0.5	0.5	60.00 ±0.00^{k}	2.42 ±0.05^{ijk}
	1.0	0	71.60 ±0.51^{h}	3.10 ±0.10^{e}
	1.0	0.25	55.60 ±0.24^{l}	2.40 ±0.00^{ijk}
	1.0	0.5	40.00 ±0.55^{o}	2.04 ±0.02^{mn}
CV(%)			**1.89**	**7.63**
LSD (5%			**1.29**	**0.22**

Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=5).CV = Coefficient of variation, LSD = Least Significant Difference, SD= Standard Error.

respectively after 30 days of acclimatization (Table 7). Loss of some plantlets might be due to differences in the genotype in adaptation to the new environment (*ex vitro*). The less development of cuticle under *in vitro* condition and the drop in relative humidity from near 100% in the culture vessels to much lower values in the greenhouse

Table 6. Number roots per shoots as affected by different concentration and combination of Auxins (IBA and NAA).

PGR concentration (mg/L)		Number of roots per shoot
IBA	NAA	
0	0	1.80±0.14[i]
0	0.25	-
0	0.5	-
0.25	0	5.43±0.08[a]
0.25	0.25	4.07±0.12[d]
0.25	0.5	3.31±0.06[e]
0.5	0	4.53±0.08[c]
0.5	0.25	4.80±0.11[b]
0.5	0.5	2.87±0.13[f]
1.0	0	3.50±0.05[e]
1.0	0.25	2.47±0.06[g]
1.0	0.5	2.07±0.12[h]
LSD (5%)		0.25
CV (%)		12.28

Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=15). LSD=Least Significant Difference, CV= Coefficient of variation, SD= Standard Error and n=number of samples, '-' indicates no response.

Table 7. Survival rate of plantlets derived from *in vitro* regeneration through direct organogenesis of three Jatropha accessions during acclimatization.

Accession	Total no. of plants acclimatized	No. of plants survived	No. of died plants	% of survived Plants	% of died plants
Adami Tulu	15	10	5	66.67	33.33
Metema	15	11	4	73.33	26.67
Shewa Robit	15	13	2	86.67	13.33

might result inexcessive water loss and death (Biradar et al., 2009). The current result is in agreement with the report of Jeevan et al. (2013) who declared 87% greenhouse acclimatization potential of *in vitro* generated Jatropha cultures. There were no observable variations with respect to morphological and growth characteristics between *ex vitro* sown parent plants and *in vitro* raised plants in pots.

Conclusion

This study provided optimal protocol for i*n vitro* mass propagation of Jatropha accessions viz. Metema, Adami Tulu and Shewa Robit accession through direct organogenesis from nodal explant. The present study concluded that best shoot induction from nodal explant was obtained from MS medium containing 1 mg/L BAP and 0.5 mg/L IBA for all the three accessions. MS media supplemented with combination of 0.5 mg/L BAP and 0.5 Kn mg/L followed by 0.5 mg/L BAP is sufficient for maximum shoot multiplication and growth parameters for the three accessions. On the other hand, the longest shoot length for Metema and Adami Tulu accession were obtained from both MS medium containing 0.5 mg/L Kinetin and PGRs free MS medium with no significant difference. That means free MS media can also be used for shoot elongation. Among various concentration and combination of auxin tested, half MS medium supplemented with 0.25 mg/L IBA followed by 0.5 mg/L IBA with 0.25 mg/L NAA were best for all root induction and growth parameters for the three accessions. *In vitro* induced shoots were well rooted and successfully established in green house environment. Generally, in this study an efficient direct organogenesis protocol was developed for Jatropha and this technique can be used

as guidelines for improving *in vitro* mass propagation of the crop.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effects of genotype and plant growth regulators on callus induction in leaf cultures of *Coffea arabica* L. F1 hybrid

Mwaniki W. I.[1*], Lubabali A. H.[2], Asava K. K.[2], Agwanda C. O.[3] and Anami S. E.[1]

[1]Institute Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology P. O. Box 50957-00200 NBI, Juja, Kenya.
[2]Coffee Research Institute, KALRO, P. O. Box 4-00232 Ruiru, Kenya.
[3]Centre for Agriculture and Biosciences International (CABI), P. O. Box 633-00621, Nairobi, Kenya.

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***Coffea arabica*, F1 hybrid variety, Ruiru 11 is a highly sought after crop in Kenya due to its alleviated resistance to Coffee Berry Disease and Coffee Leaf Rust coupled with high yield capacity and good cup quality. Access to the variety's planting materials is limited due to challenges with difficulty in propagation using conventional methods of seed and vegetative propagation; and somatic embryogenesis is regarded as a suitable alternative propagation method. Therefore, the current study aimed to establish an induction protocol in F1 composite hybrid Ruiru 11. The current study investigated the effects of genotype and plant growth regulators, auxins and cytokinins, on induction of embryogenic callus in two composite genotypes of *C. arabica* L. F1 hybrid variety Ruiru 11. Leaf explants from the F1 hybrid were cultured on half-strength Murashige and Skoog (MS) media supplemented with varied concentrations and combinations of plant growth regulators. Callus formation was evaluated weekly until the 60th day. Genotypic effects were assessed based on the difference on callus induction rates, biomass fresh weights and callus formation. The genotypes tested showed highest callus induction 88% (Code 71) and 100% (Code 93) with respect to the formation of embryogenic calli. Highest fresh weight was obtained at 0.973 ± 0.011 g in Code 71 and 0.649 ± 0.03 g in Code 93 in MS media supplemented with 2,4-D + BAP (0.53 + 0.11 µM). The observed results are useful in formulating the best growth regulator concentration suitable for mass *in vitro* propagation genotypes of Arabica coffee hybrid Ruiru 11 through callus induction *in vitro* of leaf explants.**

Key words: Somatic embryogenesis, callus, auxins, cytokinin, *in vitro*.

INTRODUCTION

Coffee is one of the most important commodities in the international trade and is cultivated in almost 80 countries where 70% of the total global production is by smallholder farmers (Bunn et al., 2015). In Kenya, the

*Corresponding author. E-mail: mwanikiirene95@gmail.com.

coffee contributes about 1% to the national GDP and 8% of the total agricultural export earnings after tea and horticulture (FAO, 2013). The industry supports around 700, 000 households representing 4.2 million Kenyans (FAO, 2013). Despite this, coffee production has been on the decline falling from an average of 1.5 million bags in the 1970s to 790, 000 bags by 2018 (ICO, 2019). In light of the significance of the commodity to the Kenyan economy, substantial efforts have been directed towards the revitalisation of the coffee sector. The focus entails increase coffee productivity through the adoption of large-scale adoption of high yielding disease resistant F1 hybrid coffee variety Ruiru 11.

Ruiru 11 is a composite hybrid that comprises of about 60 F1 hybrids and each is derived from a cross between several mother plants (catimor) and genetically similar males (Kathurima et al., 2010). The variety combines resistance to coffee berry disease and coffee leaf rust alongside high yield capacity, fine cup quality and compact growth. Adoption of the variety therefore not only promotes increase in production but also reduces cost of production by up to 30% due to resistance to coffee berry disease (CBD) and coffee leaf rust up (KALRO, 2019; Gichimu et al., 2013). Besides, the compact nature of the variety allows for higher density planting of 2,500 to 3000 trees per hectare thereby facilitating increased production with a yield range of 2.5 to 3 tones of clean coffee per hectare under normal management. (Gichimu et al., 2013) compared to other commercial varieties that yield at about 300 kg- 2.0 tons per ha per annum (KALRO, 2019; Gichimu et al., 2013).

Currently, production of the planting materials for the variety relies on cost-intensive conventional methods including hand pollination for hybrid seed production and vegetative propagation through cuttings. The conventional propagation methods of artificial hand pollination and cloning through rooted cuttings have proven insufficient in meeting demands for coffee planting materials in Kenya due to several limitations including the cumbersome nature of the methods, high dependence of the methods on weather conditions, high cost of labour, increased risk of spreading pests and diseases during transportation and low success rate with respect to rate of fruit set and rooting of cuttings (Berthouly and Etienne, 1999). In addition, the methods are inefficient in maintaining the genetic fidelity of the composite hybrid variety (Gichimu et al., 2012).

Several reports exist on the successful regeneration of plants using somatic embryogenesis techniques (Ducos et al., 2007; Mohebodini et al., 2011). In coffee, two methods of somatic embryogenesis (SE) have been studied, direct somatic embryogenesis (DSE), (Hudson, 2015) and indirect somatic embryogenesis (ISE), The ISE approach is preferred for mass propagation in tissue culture (Ducos et al., 2007; Jayaraman et al., 2014; Ahmed and Disasa, 2013) due to the production of a high number of somatic embryos per gram of callus compared to DSE. Despite the success associated with ISE, reports give caution on the specificity of genotype, and its interaction with exogenous plant growth regulators supplemented in the nutrient medium in coffee (Nic-Can et al., 2015). Therefore, this requires empirical tests to optimize *in vitro* callus induction protocols specific to the F1 composite hybrid, Ruiru 11. The current research was undertaken with these facts in mind and had, as an objective, to investigate the effects of genotype and different combinations of exogenous plant growth regulators on callus induction on two genotypes of *C. arabica* F1 hybrid, Ruiru 11.

MATERIALS AND METHODS

Plant materials

The study was conducted at the Plant Tissue Culture Laboratory at the Coffee Research Institute (Ruiru, Kenya). Twenty seedlings of F1 hybrids Code 71 and Code 93 (Table 1) were used for the study. The seedlings were raised in the greenhouse for a period of 3-6 months.

Establishment of callus cultures

The third leaf-pairs were excised from the donor plants and placed in a beaker containing tap water and transferred to the tissue culture laboratory. The leaves were surface sterilized on both sides using cotton wool dipped in dilute Teepol detergent and rinsed under running tap water. In a sterile laminar flow hood, further surface sterilization was done using 20% commercial bleach (JIK) which contain 3.85% (w/v) sodium hypochlorite for 15 min and rinsed thrice using sterile distilled water. The leaf explants were subsequently immersed in 70% ethanol and rinsed three times with sterile distilled water (Hudson, 2015). These were then dissected into leaf squares measuring approximately 1 cm × 1 cm, excluding the main vein and edges (lateral apical and basal portions). The explants were inoculated in ½ Murashige and Skoog (MS) media culture media with the adaxial-side down in culture vessels containing three leaf discs per vessel.

Effects of plant growth regulators

Half-strength MS media was supplemented with different plant growth regulators (2, 4-D, IBA, BAP, and KIN) (Table 2), concentrations, sucrose (30 g/L), 100 ml/L inositol, 30 mg/L cysteine-HCL and 40 g/L gelrite. Media pH was adjusted to 5.7 (using 1n NaOH and 1n HCL). The media was sterilized at 121°C under 1.1 psi for 15 min prior to culture. Control experiments where MS media was not supplemented with plant growth regulators and inoculated with explants were used. The culture vessels containing explants were sealed and incubated in dark growth room at 25 ± 2°C. The frequency of callus induction was recorded every two weeks and calculated after 60 days, as

$$\text{Callus induction (\%)} = \frac{\textit{Total Number of explants with callus induction}}{\textit{total number of explants inoculated for each treatment}} \times 100$$

Callus characteristics and growth parameters

The callus characteristics were graded based on morphology,

Table 1. Pedigree on Code 71 and Code 93 *C. arabica* Ruiru 11.

Coffee hybrid	Pedigree	
Code 71	SL28	[(N39 x HT) x (SL28 x RS)] x Catimor
Code 93	SL28	[(SL34 x RS) x HT)] x Catimor

RS = Rume Sudan, HT = Híbrido de Timor.
Source: Gichimu et al. (2014)

Table 2. Callus Induction from leaf explants of *C. arabica* Code 71 in MS media supplemented with different concentration of auxins and cytokinin.

Plant growth regulator	Concentration (µM)	Degree of callus formation	Duration of calli initiation (weeks)	Color and morphology of callus	% Callus induction	Callus FW (g ± SE)
	0	-	-	NC	-	-
2,4-D	0.11	++	6	FW	78	0.178±0.0256^{c}
	0.22	++	6	FW	85	0.320±0.006^{b}
	0.33	++	5	FW	88	0.490 ±0.013^{a}
	0.44	++	5	FW	50	0.0962 ±0.011^{d}
	0.53	++	5	FW	45	0.0672 ±0.002^{d}
2,4-D + BAP	0.11 + 0.56	+++	5	FW	68	0.237±0.017^{c}
	0.22 + 0.45	+++	5	FW	80	0.294±0.023^{b}
	0.33 + 0.34	+++	4	FW	75	0.298±0.011^{b}
	0.44 + 0.23	++++	4	FW	76	0.593±0.030^{a}
	0.53 + 0.11	++++	4	FW	88	0.649±0.026^{a}
2,4-D + KIN	0.11 + 0.54	++	6	CW	53	0.088±0.002^{bc}
	0.22 + 0.43	++	5	CW	60	0.080±0.052^{a}
	0.33 + 0.32	+++	5	FW	68	0.238±0.016^{a}
	0.44 + 0.21	+++	5	FW	73	0.200±0.002^{b}
	0.53 + 0.11	+++	5	FW	76	0.037±0.013^{b}

NC= no callus formed, + = very poor, ++ = poor, +++ = good, ++++ = very good. Means that do not share a letter are significantly different at p =0.05 determined under Fischer's LSD test. FW= Friable white, CW – compact-white, SE= Standard error.

callus score, and color. Callus morphology was characterized after 60 days of culture inoculation based on two characteristics: (i) friable (embryogenic) and (ii) compact (non-embryogenic) as shown in Figure 1. The study evaluated callus score based on growth viability under five categories: NC= no callus, + = very poor, ++ = poor, +++ = good and ++++ = very good for each of the plant growth regulator treatment. Visual analysis of callus induction period was determined as time taken to initiate visible callus from the leaf explants. Callus weights were measured on a precision scale (g) in a sterile laminar flow hood as described by Balbaa et al. (1974). For growth curve, analysis of explant cultures was initiated on the first day of inoculation (day 0). Subsequently, analyses were done at intervals of 7 days until the 70th day. The growth rate was assessed as described by Dung et al. (1981).

Effect of genotype on callus induction

The two genotypes, Code 71 and Code 93, were inoculated in MS Media to test for the callogenesis response. Effects were assessed based on the formation of embryogenic and non-embryogenic calli and callus induction rates (%).

Regeneration

After an incubation period of 45 days for proliferation of embryogenic calli obtained, calli was transferred to half-strength MS regeneration media maintained in a growth room under 16 h photoperiod and 8 h dark cycle. Plantlets with well-developed shoot and root systems were carefully washed off-culture media and transplanted into pots and covered with transparent plastic lids to maintain humidity.

Statistical analysis

The experiments were laid out in completely randomized design and repeated three times with 20 replicates. The results were assessed by a standard analysis of two-way ANOVA variance (Fischer'sTest) using Minitab® 17.1 Software. Each genotype was

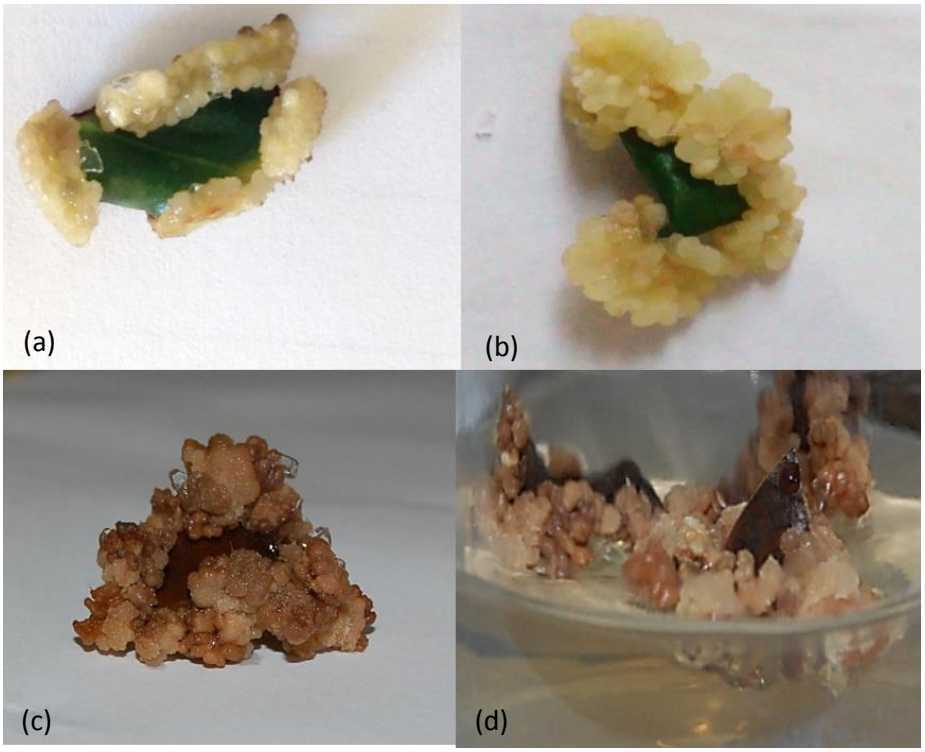

Figure 1. (a) non-embryogenic calli as observed in leaf explants Ruiru 11 *cv*. (b) embryogenic calli as observed in leaf explants Ruiru 11 *cv*. (c & d) Browning of calli of leaf explants of Ruiru 11 cv. as a result of prolonged periods in culture.

assessed individually. To test the genotypic differences on Code 71 and Code 93 two-sample t-test was performed.

RESULTS

Callus induction

Callus induction was assessed in leaf cultures of Code 71 and Code 93. Calli was induced within 10 days of inoculation. Cultures in half-strength MS media supplemented with no plant growth hormones did not induce callus in both codes.

Plant growth regulators (PGR) used singly, auxin 2,4-D induced callus whereas, auxin IBA and cytokinin (BAP and KIN) did not induce callus even after 40 days of culture. Highest induction frequency was observed for 2,4-D (0.53 µM) in Code 93 at 97% (Table 3) and 2,4-D (0.33 µM) in Code 71 at 88% (Table 2). The induction frequency, however, varied within concentrations. Low concentration levels of 2,4-D in Code 71 was observed to have increased induction rates, whereas higher concentration levels recorded reduced induction rates (Table 2). The opposite was true for Code 93, which recorded increased induction rates with increased concentration levels of 2,4-D (Table 3). The results suggest that genotype plays an important role in callus induction. Both the type of PGR and the concentration of the same are therefore important in callus induction in Arabica coffee hybrids.

Auxin and cytokinin combinations significantly affected callus induction in both Code 93 and Code 71. 2,4-D + BAP overall recorded highest induction rates in both Code 71 (Table 2) and Code 93 (Table 3). 2,4-D + BAP (0.53 µM + 0.11 µM) resulted in highest induction rates at 100% in Code 93 (Table 3) and 88% in Code 71 (Table 2). The combination also reduced the duration required to observe peak callus in Code 71 by one week. The study also observed that higher concentration levels of auxins (2,4-D and IBA) combined with lower concentrations of cytokinins (BAP and KIN) improved induction rates in both Code 93 (Tables 3 and 5) and Code 71 (Tables 2 and 4).

Callus characteristics

Preliminary callus structures were visually observed at the cut edges of the leaf discs after 7 days of culture and proliferation was observed for a period of 8 weeks (Figure 1a and b). A white callus morphology was recorded across all treatments in Code 71 and Code 93. The color of resultant calli did not affect proliferation rates across all treatments.

Table 3. Callus Induction from leaf explants of *C. arabica* Code 93 in MS media supplemented with different concentration of auxins and cytokinin.

Plant growth regulator	Concentration (µM)	Degree of callus formation	Duration of calli initiation (weeks)	Color and morphology of callus	% Calli Induction	Callus FW (g ±SE)
2,4-D	0	-	-	NC	-	-
	0.11	++	4	FW	75	0.190±0.01[d]
	0.22	+++	4	FW	88	0.394±0.008[c]
	0.33	+++	3	FW	86	0.636±0.025[b]
	0.44	++++	3	FW	96	0.919±0.023[a]
	0.53	++++	3	FW	97	0.961±0.02[a]
2,4-D+ BAP	0.11 + 0.56	+++	4	FW	75	0.196±0.006[d]
	0.22 + 0.45	+++	4	FW	80	0.271±0.022[c]
	0.33 + 0.34	+++	3	FW	93	0.402±0.033[b]
	0.44 + 0.23	++++	3	FW	100	0.973±0.011[a]
	0.53 + 0.11	++++	3	FW	100	0.971±0.017[a]
2,4-D + KIN	0.11 + 0.54	++	5	CW	50	0.280±0.015[a]
	0.22 + 0.43	++	5	CW	55	0.303±0.011[a]
	0.33 + 0.32	+++	4	FW	63	0.279±0.011[ab]
	0.44 + 0.21	+++	4	FW	73	0.304±0.012[a]
	0.53 + 0.11	+++	4	FW	76	0.249±0.012[a]

NC= no callus formed, + = very poor, ++ = poor, +++ = good, ++++ = very good. Means that do not share a letter are significantly different at p =0.05 determined under Fischer's LSD test. FW= Friable-white; CW= Compact-white, SE= Standard error.

Table 4. Callus Induction from leaf explants of *C. arabica* Code 71 in MS media supplemented with different concentration of auxins and cytokinin.

Plant growth regulator	Concentration (µM)	Degree of callus formation	Duration of calli initiation (weeks)	Color and morphology of callus	% Calli formation	Callus FW (g ±SE)
IBA+ BAP	0.10 + 0.56	++	6	CW	65	0.124±0.022[d]
	0.20 + 0.45	++	6	CW	68	0.183±0.028[c]
	0.30 + 0.34	++	6	CW	69	0.258±0.012[ab]
	0.41 + 0.23	++	5	CW	75	0.304±0.011[a]
	0.51 + 0.11	++	5	CW	71	0.249±0.012[b]
IBA + KIN	0.10 + 0.54	++	6	CW	61	0.123±0.022[c]
	0.20 + 0.43	++	6	CW	68	0.190±0.02[b]
	0.30 + 0.32	+++	5	CW	60	0.236±0.018[b]
	0.41 + 0.21	+++	5	CW	75	0.342±0.045[a]
	0.51 + 0.11	+++	5	CW	81	0.248±0.048[b]

NC= no callus formed, + = very poor, ++ = poor, +++ = good, ++++ = very good. Means that do not share a letter are significantly different at p =0.05 determined under Fischer's LSD test. FW= Friable-white; CW= Compact-white, SE= Standard Error.

Media supplemented with 2,4-D induced good friable callus (Figure 1b) formation (++) across all treatments in Code 71 (Table 2) and Code 93 (Table 3). Higher concentration levels of 2,4-D combined with lower concentration levels of BAP induced very good friable calli (++++) in Code 71 and Code 93 whereas, lower levels of 2,4-D and higher levels of BAP recorded good friable callus formation (+++) in Code 71 (Table 2) and Code 93 (Table 3). On the other hand, IBA combined with KIN and BAP induced compact calli (Figure 1a) across all treatments in Code 71 (Table IV) and Code 93 (Table 4). The browning of callus was observed after extended

Table 5. Callus Induction from leaf explants of *C. arabica* Code 93 in MS media supplemented with different concentration of auxins and cytokinin.

Plant growth regulator	Concentration (µM)	Degree of callus formation	Duration of calli initiation (weeks)	Color and morphology of callus	% Calli induction	Callus FW (g ±SE)
IBA+BAP	0.10 + 0.56	++	5	CW	60	0.280±0.015[a]
	0.20 + 0.45	++	5	CW	64	0.302±0.011[a]
	0.30 + 0.34	++	4	CW	68	0.279±0.011[ab]
	0.41 + 0.23	++	4	CW	75	0.304±0.012[a]
	0.51 + 0.11	++	4	CW	70	0.249±0.012[b]
IBA+ KIN	0.10 + 0.54	++	6	CW	61	0.273±0.015[b]
	0.20 + 0.43	++	6	CW	68	0.291±0.013[ab]
	0.30 + 0.32	+++	5	CW	58	0.274±0.015[b]
	0.41 + 0.21	+++	5	CW	73	0.341±0.044[a]
	0.51 + 0.11	+++	5	CW	80	0.248±0.009[b]

NC= no callus formed, + = very poor, ++ = poor, +++ = good, ++++ = very good. Means that do not share a letter are significantly different at p =0.05 determined under Fischer's LSD test. FW= Friable-white; CW= Compact-white, SE= Standard error.

periods in culture, which could indicate the onset of cell death (Figure 1 c and d).

Callus induction time

The shortest period for maximum callus proliferation in Code 71 was recorded in MS media supplemented with 2,4-D + BAP (Table 2) and Code 93 (Table 3). 2,4-D recorded increased initiation time with a difference of 5-6 weeks in Code 71 (Table 2) but, reduced initiation time (3-4 weeks) in Code 93 (Table 3). These observations indicate that PGRs treatments had an effect both on the time to and rate of callus proliferation. Auxin-cytokinin combinations (2,4-D, IBA) and (BAP and KIN) recorded longer initiation periods in Code 71 and Code 93. Time is a critical factor in the determination of efficient induction protocols. However, accompanying factors including biomass, callus morphology, and induction frequency, are important.

Biomass growth measurement

Results from fresh weight measurements indicated a significant difference in weights in embryogenic calli between PGR treatments. ANOVA analysis indicated that 2,4-D (0.33 µM) recorded highest fresh weight (0.490 ±0.013 g FW) in Code 71 (Figure 3) and 2,4-D (0.53 µM) recorded highest weight (0.961±0.02 g FW) in Code 93 (Figure 3). The results show a significant difference in embryogenic response. Code 71 showed increase in biomass with increase in 2,4-D levels but a decrease in embryogenic response in high concentration levels of 2,4-D. On the other hand, Code 93 showed a general increase in embryogenic response with increased concentration levels of 2,4-D. Auxin and cytokinin combinations, 2,4-D + BAP (0.53 + 0.11 µM) recorded highest weights in Code 71 yielding 0.649±0.026 g FW (Figure 2a) and in Code 93, (0.44 + 0.23 µM) recorded highest yield at 0.973±0.011 g FW (Figure 2a). 2,4-D + KIN (0.33 + 0.32 µM) recorded highest weights in Code 71 of 0.238±0.016 g FW (Figure 2b). The combination of 2,4-D + KIN (0.44 + 0.32 µM) yielded 0.304±0.012 g FW in Code 93 (Figure 2b).

Callus growth curve

The callus growth curve is necessary to identify the suitable point of transfer in coffee tissue culture. The growth curve of calli indicated three distinct phases: lag phase, exponential phase, and linear phase (dos Santos and de Souza., 2016; Daffalla et al., 2019). Lag phase and exponential phase represent cell growth ideal for cell division and cell multiplication of callus. Linear phase is characterized by reduced cell division (dos Santos and de Souza, 2016). Prolonged linear phase promotes phenolic compound production, which is not amenable for callus proliferation. This may explain the browning observed in cultures. Figure 4 indicates growth curves recorded on embryogenic calli obtained from 2,4-D + BAP (Figure 4b) 2,4-D (Figure 4a) and 2,4-D + KIN (Figure 4c) in Code 71 and Code 93. Dedifferentiation of leaf calli was evident through a lag phase from the day of inoculation to the 14th day of culture inoculation; exponential phase from the 21st day to the 42nd day of exposure and linear phases from the 42nd day of culture. The growth curves showed a peak in growth from the 28th day to the 42nd day with a subsequent decrease in

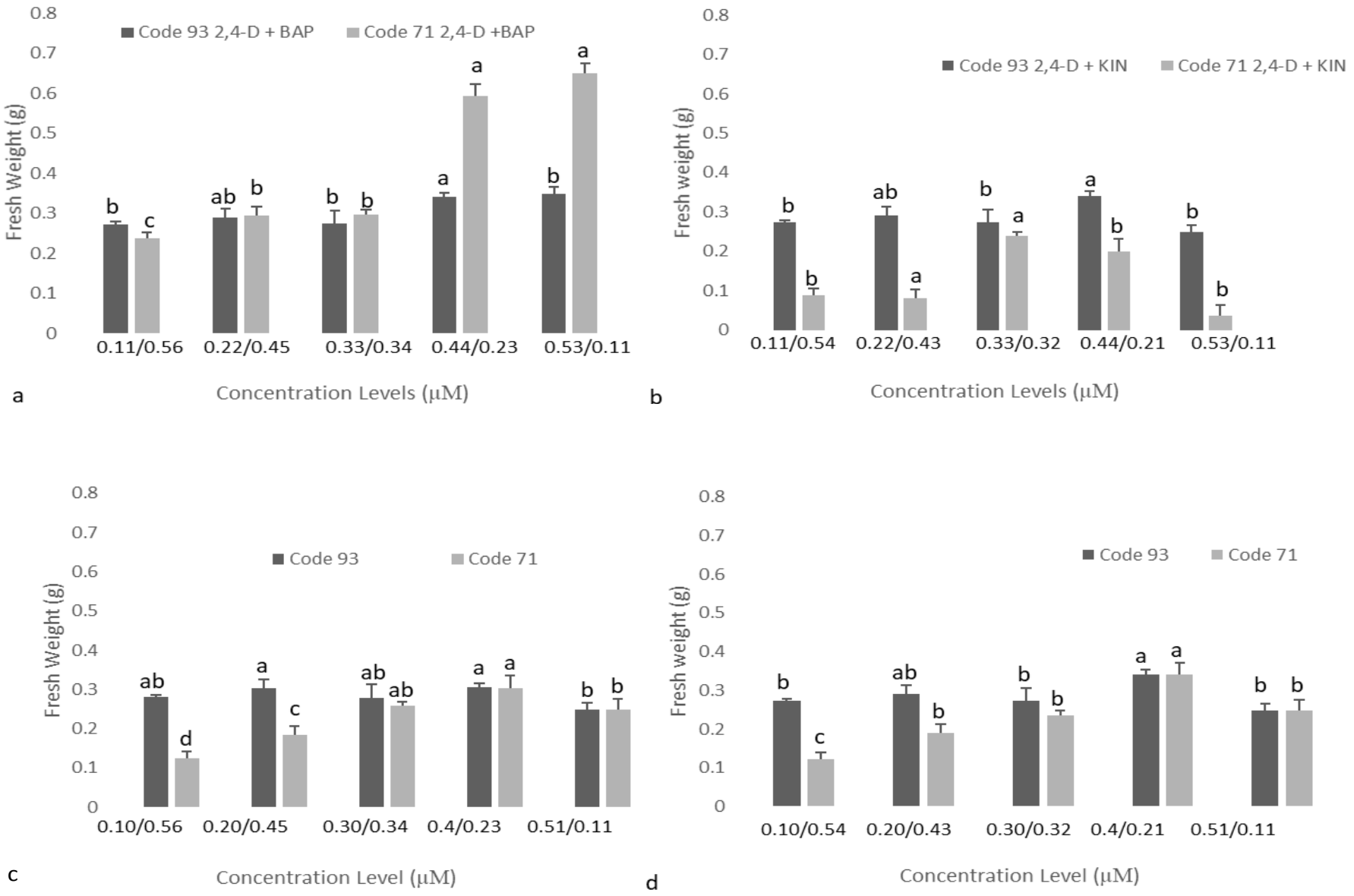

Figure 2. Effects of 2,4-D + BAP (a) and 2,4-D + KIN (b), IBA + BAP (c) and IBA + KIN (d) on fresh weight of friable and compact calli induced in Code 71 and Code 93 Ruiru 11 *C. arabica. N=60.* Means that do not share a letter are significantly different at p =0.05 determined under Fischer's LSD test.

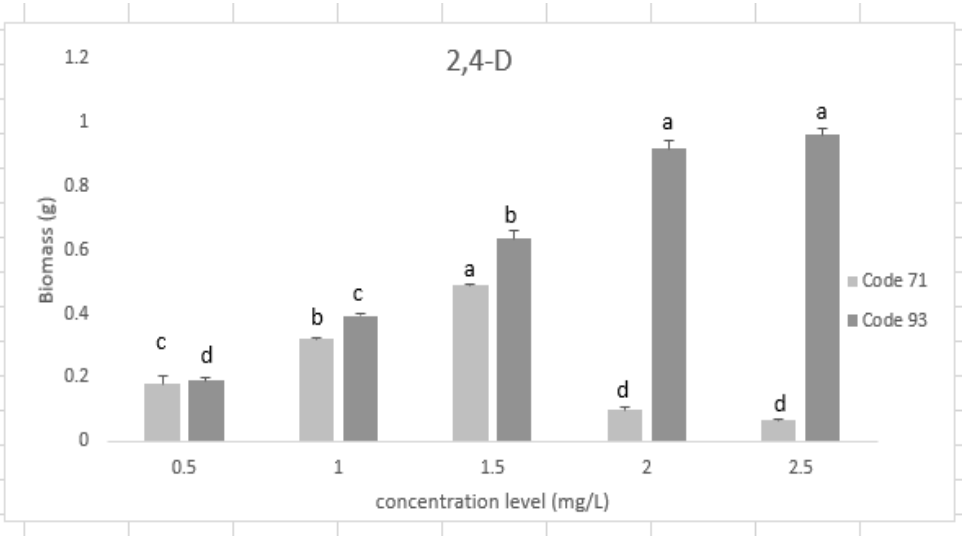

Figure 3. Effect of 2,4-D on fresh weight of calli induced in Code 71 and Code 93 Ruiru 11 cv. N=60. Means that do not share a letter are significantly different at p = 0.05 determined under Fischer's LSD Test.

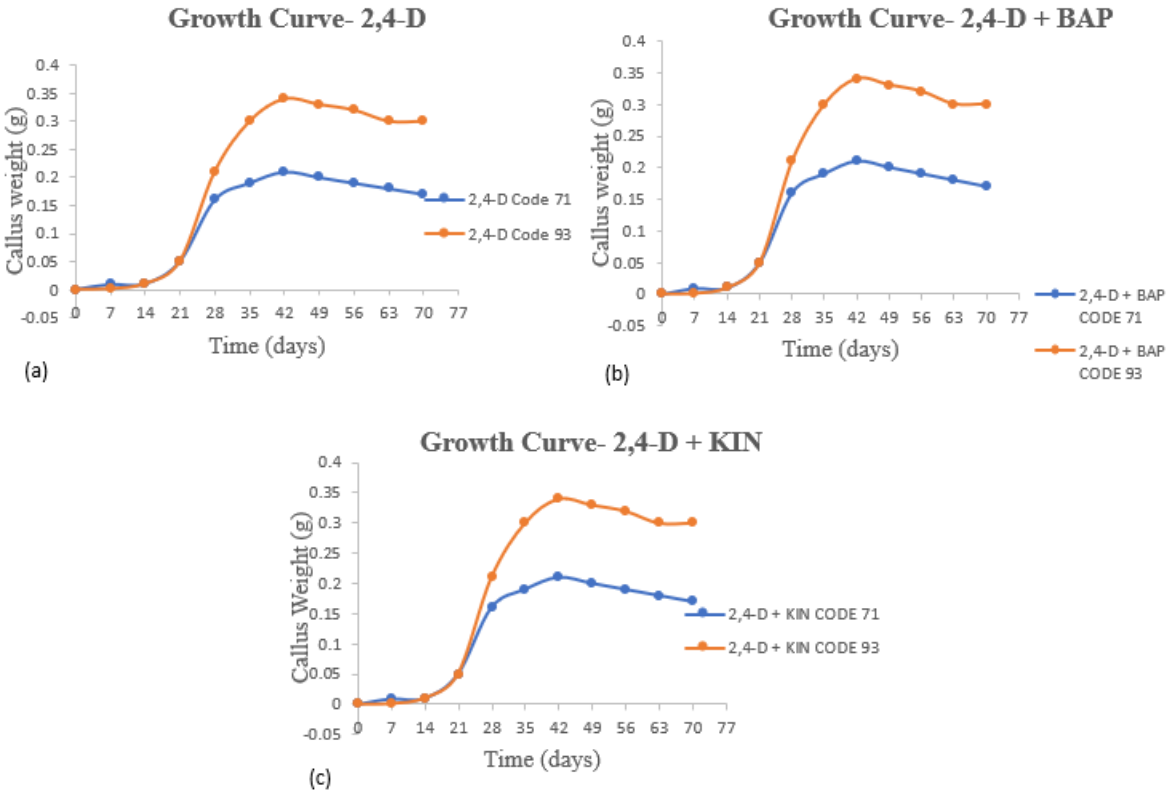

Figure 4. Growth curves of calli of Ruiru 11 cv. Code 71 and Code 93 on different plant growth regulators (a) (b) (c). Growth was determined after every seven days of inoculation.

biomass. The prolonged linear phase suggests that a decrease in growth rate results from the scarcity of nutrients or drying of solidifying agent or accumulation of toxic substances (Daffalla et al., 2019). The results,

therefore, suggest that transfer to new growth media is necessary to increase survival rates (cell viability) and sustain callus growth after the 42nd day.

Genotype

Genotype played a significant role in callus induction in cultures. The study observed varying responses in embryogenic calli induction in Code 71 and Code 93. Code 93 recorded callus induction at a 50-100% rate in the different plant growth regulators supplemented in MS media (Tables 3 and 4) whereas, Code 71 induced at a range of 45-88% (Tables 2 and 4). 2,4-D (0.53 µM) in Code 93 showed higher mean difference in embryogenic response at 0.961 g FW (SD 0.0913). By comparison Code 71 under similar treatment was observed to have numerically lower means at 0.0672 g FW (SD 0.00182). According to the t-test analysis, the results showed significant difference in means derived for Code 71 (Mean = 0.06721; SD = 0.00814) and Code 93 (Mean = 0. 9612; SD = 0.0913) under conditions p-value (0.000) and t-value (-43.63). The difference in response rates explanation to this could be attributed to genealogy influences.

Regeneration

Regeneration of Code 71 and Code 93 was carried out on half-strength MS media supplemented with proline 4 mg/L, sucrose 20 g/L and GA_3 at 3 mg/L. Efficiency rate in formation of well-developed shoot and root system was determined as a percentage. Code 71 regenerated 68.2% and Code 93 at 75.3%. Two-way ANOVA analysis indicated regeneration potential for Code 71 at 523.8 ± 0.25 and Code 93 at 706.5 ± 0.11. The results show variation in Code interaction with half-strength MS media in regeneration potential at probability 0.05.

DISCUSSION

Callus induction

From the results observed on the control experiments with no plant growth regulators in culture media, there was no callus induction in all the trials. This is expected given that both plant growth regulators are paramount in stimulating growth and differentiation in cells and tissues under physiological process. Callus induction is a physiological process that requires the influence of plant growth regulators for cell division and differentiation. Auxins are effective in stimulation of cell elongation and vascular differentiation while cytokinins are critical in stimulation of cell division (Gaspar et al., 1996; Kumar et al., 2016). Gaspar et al., (1996) elucidates that effective callus induction is as a result of positive interaction between endogenous PGR and exogenous PGR. Exogenous PGR biological activity can be equivalent to or be in excess of endogenous PGR to influence physiological activity including callus induction. The present observations, therefore, support the results obtained hitherto by other researchers in coffee and other crop species. Similar results were obtained by Ahmed and Disasa, (2013) in *C. arabica* leaf explants and dos Santos and de Souza (2016) in *Capsicum annum*.

The PGR used singly, auxin 2,4-D induced callus in all treatments in Code 71 and Code 93. This agrees with and reemphasize the observations by Maciel et al., (2016) in *Coffea arabica* L., Rashmi and Trivedi (2014), in *Nerium odorum* (Apocynaceae), Dos Santos and De Souza (2016) in *Capsicum annum*, Gopitha et al. (2010) in *Saccharum officinarum* (Sugarcane), and Lee et al. (2011) in *Morus alba* (Mulberry) all whom observed embryogenic response in callus formation using 2,4-D.

Unlike auxins, the single treatment with cytokinins (KIN and BAP) did not yield any embryogenic response. This is expected from the observation because Gaspar et al., (1996) points to the fact that cytokinins are suitable for cell division, with BAP and KIN being the most commonly used. The authors further observed that cytokinins are effective in callus formation when combined with auxins; this was observed to be true for the coffee genotypes investigated through this study. The reason is that cell division is a joint action that requires a synergistic relation between auxins and cytokinins (Varshney et al., 2013). In plant cells, for auxins to be effective, the PGR has to be protected from oxidative denaturization through molecular conjugation enabling storage and consequential, gradual release for enzymatic action (Gaspar et al., 1996).

The level of concentration of the respective PGRs was observed to have an important impact on the rate of callus induction, with different genotypes displaying different reaction patterns with low concentration levels of 2,4-D (0.11, 0.22 and 0.33 µM) resulting in higher induction rates in code 71 whereas a linear increase in induction rates was observed in Code 93 with increased concentration levels. This is in line with the observations by Molina et al. (2002) and Rezende et al. (2012) in *C. arabica*. Overall, Code 93 recorded highest induction rates compared to Code 71 which indicate that induction rates are strongly influenced by genotype which, supporting the observations by Jiménez (2001) that embryogenic response to PGR is directly related to genotype.

Auxin and cytokinin combinations significantly affected callus induction in both Code 93 and Code 71. Similar observations have indeed been made in other studies including Gatica-Arias et al. (2008), Maciel et al. (2016) and Aga and Khillare (2017) in *C. arabica* callus induction using auxin and cytokinin combinations.

Auxins (2,4-D and IBA) combined with cytokinin (BAP and KIN) obtained callus formation across all treatments

in Code 71 and Code 93. However, difference in embryogenic response was observed within the treatments. The differences were specific to auxin and cytokinins concentration and type along with genotype. The present study obtained highest induction rates in 2,4-D + BAP in both Code 71 and 93. Contrary, studies by Etienne et al., (2018), Gatica-Arias et al., (2008) report of best callus induction was obtained in treatments supplemented with 2,4-D + KIN. For PGR to be effective, Gaspar et al., (1996) elucidate that endogenous PGR interacts with exogenous PGR by which exogenous PGR biological activity can either be equivalent to or in excess to endogenous PGR. The interaction with endogenous PGR is specific, and cell and tissue responses greatly rely on plant species, the genotype of species and explant source. In most cases, auxin and cytokinin interaction can either be synergistic or antagonistic, whereby, auxins inhibit cytokinin action and vice versa. This is reflected by the induction competence between Code 71 and Code 93.

Callus morphology

Assessment of callus induction in tissue culture is mandatory to determine formation of embryonic and non-embryonic calli. The reason is because somatic embryogenesis can only be determined as a result of differentiation of callus cells into embryos (Ikeuchi et al., 2013). Embryogenic calli are preferred due to characteristic loosely aggregated cells of low densities which have high cell viability for embryogenesis in coffee (Leva et al., 2012; dos Santos and De Souza, 2016). The present study obtained embryogenic calli in auxin 2,4-D and auxin-cytokinin, 2,4-D + BAP across all treatments in Code 71 and Code 93. Similar observations are reported by Molina et al. (2002), Maciel et al. (2016) and Aga and Khillare (2017) in *C. arabica*; Durrani et al. (2017) *Solanum* spp, and Gopitha et al. (2010) *Saccharum officinarum*.

Non-embryogenic calli was also observed in treatments supplemented with 2,4-D + KIN, IBA combined with BAP and KIN across all treatments (Code 71 and Code 93). Contrary, Etienne et al., (2018) reported inducing embryogenic calli using 2,4-D + KIN (0.11 + 0.54 µM) while, Gatica-Arias et al., (2008) reported the same results at 2,4-D + KIN (4.52 +18.56 µM) in *C. arabica* leaf explants. Ahmed snd Disasa, (2013) on *C. arabica* leaf explants callus induction using IBA + KIN reported induction of embryogenic call whereas, Mohajer et al. (2012) on *Onobrychis sativa*, and Wahyuni et al. (2017) on *Justicia gendarussa* reported non-embryogenic callus induction in media supplemented with IBA + BAP. The results infer that plant growth regulators and genotype of plant can influence varying callus formations-embryogenic and non-embryogenic calli.

Code 71 and Code 93 are obtained from similar coffee variety, Ruiru 11, and similar explant, but, presented varying responses based on formation of embryogenic and non-embryogenic. This is attributed to their genotypic difference (Table 1). Jiménez (2001) suggests that embryogenic and non-embryogenic competence in callus formation in similar explants from genetically identical cells and tissues respond differently to varying stimuli which could also be the case with respect to Codes 71 and 93 in this study.

The present study observed varying callus score in treatments supplemented with auxins and cytokinins. Knowledge on the influence of degree of callus formation in relation to callus induction efficiencies is obscure. However, this study observed varying callus scores in based on plant growth regulator and genotype. The observations indicate that callus score is influenced by plant growth regulator used singly or in combination with auxin-cytokinin recording good calli formation in Code 71 and Code 93. Also, observations suggest that callus score is dependent on genotype of coffee species.

Time is a critical factor when optimizing micropropagation protocols for high-frequency somatic embryogenesis for large-scale propagation in coffee (dos Santos and de Souza, 2016). As such, shorter induction periods are superlative compared to longer induction periods (Samson et al., 2006). With regard to callus induction time, the results suggest that time taken to induce callus is influenced by genotype as well as PGR and concentration level. This parameter is often coupled with embryo formation yield, which should emphasize on high-frequency somatic embryogenesis.

Callus growth curve

Investigation on callus growth curve is paramount to determine the deceleration phase (Dos Santos and De Souza, 2016). A growth curve encompasses phases that include lag phase, exponential phase, linear phase, and deceleration phase. The pattern of the growth curve is dependent on plant species (dos Santos and de Souza, 2016; Daffalla et al., 2019). The present study observed three growth stages; lag, exponential, and linear phase. The growth curve of the study did not obtain the deceleration phase, but, phenolic compound production (synonymous with browning in coffee) was reported. Browning is tantamount with activation of secondary metabolites, phenolic compounds, (Ignacimuthu et al., 1999) considered to be a severe problem in indirect somatic embryogenesis where it inhibits growth resulting in reduced regeneration potential and recalcitrance (Jones and Saxena, 2013). Data in Figure 4 indicate that peak growth competence was achieved in embryogenic calli on the 42nd day with a subsequent slight deceleration in the growth curve. This peak indicates that probably optimal growth was achieved at the 42nd day, which consequently should be the ideal time to transfer calli to new media for callus proliferation hence increase survival

rate and improve on callus growth. A similar conclusion was reached by dos Santos and de Souza (2016) working on *C. canephora* calli. Indicating that genotype has no influence over callus growth curve.

Biomass yield

The embryogenic potential is determined by biomass yield, which is an important factor in coffee somatic embryogenesis. Auxin, 2,4-D used singly in Code 71 (Table 2) showed reduced biomass yield with increased concentration levels. Contrary, Code 93 (Table 3) was observed to increase biomass yield with increased concentration levels. Similar results were obtained with 2,4-D + BAP in Code 93 and Code 71 across all treatments. However, biomass yield presented no effect with induction rates across all treatments in Code 71 and Code 93. The results suggest that genotype and PGR affect biomass yield in Ruiru 11 F1 hybrids Code 71 and Code 93. This observation is important given that optimized micropropagation protocols rely on high-frequency somatic embryogenesis, which is directly related to induction rates; time taken to induce callus and biomass yield.

Therefore, the difference in embryogenic response in Code 93 and Code 71 was varied based on assessed parameters in the present study through student t-test. This further confirms the conclusion by Nic-can et al. (2015) that somatic coffee embryogenesis is highly dependent on the genotype of coffee, explant source, and type and concentration of plant growth regulator. The results obtained have shown a strong inclination on the influence of auxins and cytokinins and concentration levels on callogenesis, meaning that both PGR choice and concentration levels are an important consideration in the optimization of protocols for somatic embryogenesis in the species. The results suggest that the difference in growth response is also attributed to the diversity in the genealogy of F1 hybrid Ruiru 11 Code 71 and Code 93. Molina et al. (2002) observed that coffee genealogy plays a crucial role in embryogenic capacity in genotypes which is based on the genealogy of progenies and embryogenic response is under a strong genetic control (Orians, 2000; Molina et al., 2002). Ruiru 11 is a composite Arabica coffee that comprises of hybridization of diverse coffee species including Robusta coffee and difference in results observed for the two clones may be a reflection of the differences in the residual backgrounds of the respective progenitors for the two clones.

Conclusion

The study established that combined use of plant growth regulators 2,4-dichlorophenoxy acetic acid [2,4-D] and 2,4-dichlorophenoxy acetic acid [2,4-D] + Benzyl amino purine [BAP] are ideal induction protocol for *C. arabica* F1 hybrid, Ruiru 11. The study observes that the presence of genotypic difference with respect to response to treatments with plant growth regulators leading to differences in induction rates, response rates and callus morphology. The study recommends that adoption of the induction protocol for Code 71 and Code 93 is necessary to induce callus effectively and regenerate coffee planting materials to meet current demand. It is therefore recommended that further studies be undertaken using representative sample of codes of Ruiru 11 given that the current study had limited number of codes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Relationship between 2-acetyl-1-pyrroline and aroma in Uganda rice populations with *Oryza (barthi*, *glaberrima* and *sativa*) backgrounds

David Ocan[1,4*], Rongrong Zhang[2], Martin Odoch[4], Michael Kanaabi[3], Angele Pembele Ibanda[1], Agnes Akwero[1], Bill Williams Khizzah[4], Ephraim Nuwamanya[3], Jimmy Lamo[3], Melissa Anne Fitzgerald[2], Venea Dara Daygon[2] and Patrick Rutimbanzigu Rubaihayo[1]

[1]Department of Agricultural Production, College of Agriculture and Environmental Sciences, Makerere University, P. O. Box 7062, Kampala, Uganda.
[2]School of Agriculture and Food Sciences, University of Queensland, St Lucia, QLD, 4072, Australia.
[3]National Crops Resources Research Institute, P. O. Box 7084, Kampala, Uganda.
[4]Department of Agronomy, Faculty of Agriculture and Environment, Gulu University, P. O. Box 166, Gulu, Uganda.

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The sweet popcorn aroma conferred by 2-acetyl-1-pyrroline (2AP) is a highly economic trait of rice grain attracting premium price worldwide. This research study was conducted to determine the levels of 2AP in Ugandan rice lines with the aim of establishing a better understanding on the level and classes of 2AP and aroma phenotype. Concentration of 2AP was assayed using two-dimensional gas chromatography-time-of-flight mass spectrometry (GC × GC-TOF-MS) in tandem with sensory evaluation. Substantial variations in aroma intensity within and between the Uganda rice families were recorded. However, the levels of aroma variation were strongly influenced by the type of rice, and the breeding population it was derived from. Hence, three aroma based categories, namely, nonaromatic, moderately aromatic and highly aromatic were identified. GC with complementary sensory evaluation suggested a highly complex nature of rice aroma, as several rice lines were re-classified on the basis of this study. The 2AP contents and aroma intensity for genotypes with *O. glaberrima* were low compared to *O. sativa* and *O. barthi*. Genotypes of Supa 5, Supa 1052, Yasmin aromatic and MET 3 contained high 2AP levels whereas MET 16, MET 6, AGRA 78, AGRA 55, AGRA 41 and Sande TXD 306 exhibited moderate 2AP contents. Therefore, in developing an optimal breeding strategy aimed at improving the aroma in rice, quantitative information about 2AP and complementary sensory evaluation are a prerequisite.

Key words: Grain aroma, sensory evaluation.

INTRODUCTION

Rice (*Oryza sativa* L.) is the second most important food crop after wheat worldwide (FAOSTAT, 2017) and it provides approximately 70% of the dietary energy intake for more than two billion people (Sharma et al., 2018). Africa reportedly produces at least 23.8 million tons of rice, approximately 16.4% of the global milled rice production (FAOSTAT, 2017). In Uganda, rice productivity has increased since 1961 from 1.3 to 2.3 t/ha (Kikuchi et

al., 2015) with recently estimated total production at 261,620 tons (FAOSTAT, 2017). Even with the recent yield increase mainly due to newly released high yielding rice varieties, the country is unlikely to attain self-sufficiency in rice production by 2025 (Oort et al., 2015). Consequently, research has been focused on improving productivity at the expense of enhancing quality traits of importance to final consumers (Custodio et al., 2016; Asante, 2017).

Rice consumers have diverse preferences for extrinsic and intrinsic quality attributes of the grain (Laizer et al., 2018). With regard to intrinsic sensory attributes, the sweet taste and aromatic characteristics of rice grain are the core traits desired by the majority of consumers (Laizer et al., 2018), and the basis of aroma in rice may be classified as aromatic or nonaromatic rice. Aromatic rice is mainly defined by the concentration of 2-acetyl-1-pyrroline (2AP), a N-heterocycle produced by a mutation in the betaine aldehyde dehydrogenase (*badh2*) gene, leading to accumulation of 4 aminobutanal, its acetylation and subsequent cyclisation (Daygon et al., 2017). 2-Acetyl-1-pyrroline is a key odorant in aromatic rice, with a very low detection threshold of 0.05 µg/L (Jost et al., 2019). In fact, analysis of 2AP content in several aromatic rice lines found the existence of substantial variation in the aromatic compound content (Xie et al., 2019). Nonaromatic rice was reported to contain 2AP concentration of less than 30 ppb (Buttery et al., 1983) with the highest 2AP concentration of nonaromatic rice 5-fold less than the lowest 2AP concentration of aromatic rice (Sansenya et al., 2018). Interestingly, some aromatic rice lines have been found to have non-detectable levels of 2AP, the implication being that several other compounds are involved in rice aroma (Xie et al., 2019). In addition to 2AP, five (decanal, 2-hexanone, 2-pentylfuran, 1-hexanol, and hexanal) additional volatile compounds have been found to substantially influence the categorization of rice as aromatic and nonaromatic (Hoffmann et al., 2019). Genetic factors are considered the primary contributor towards the aroma of rice grain (Bradbury et al., 2005). In fact, Shan et al. (2015) reported the creation of fragrant rice (with high 2AP content) from a nonaromatic variety by using gene editing technologies, sequence-specific nucleases and transcription activator-like effector nucleases. Despite, the significant influence of genetics on rice grain aroma, several other factors are known to contribute towards the aromatic profile of rice, and the intensity, for example soil salinity (Poonlaphdecha et al., 2012), plant nutrition (Lei et al., 2017), agronomic practices (Goufo et al., 2010), light intensity (Mo et al., 2017) and temperature (Prodhan et al., 2017). The concentration of other biological compounds such as 1-pyrroline has been directly implicated in the biosynthesis of 2AP (Prodhan et al., 2017).

Recently, in Uganda, rice comprising of landraces and introductions are being targeted for improvement of rice aroma using conventional breeding (MAAIF, 2012). Currently, 2AP is the only compound that rice breeders can select to adjust fragrance in rice (Okpala et al., 2018). However, the relationship between the concentrations of 2AP in the different rice lines, especially the landraces was not well understood. Chakraborty et al. (2016) observed variation in the intensity of rice aroma in 84 genotypes within identical genotypes and also among the genotypes from diverse groups. Therefore, the aim of this work was to determine the concentration of 2AP for the rice germplasm collection in Uganda.

MATERIALS AND METHODS

Genetic materials used

Rice grains (300 g) from each of twenty eight rice genotypes were taken from the East African Regional Rice Research and Training Centre at the Ugandan National Crops Resources Research Institute (NaCRRI), Uganda. The selected rice genotypes included introduced and local lines (Table 1).

Preparation of the polished rice grains

Rice paddy (300 g) was submerged in 1 L distilled water and the empty kernels that floated immediately removed. The clean samples were sun dried by spreading thinly (depth < 1 cm) and evenly on a clean concrete floor for approximately 48 h until approximately 12% average moisture content measured using moisture meter was achieved. The dried grains were milled using lab test mills (Satake, Tokyo, Japan) at Tilda Rice (Uganda) Limited. Fifty grams (50 g) of milled rice samples were vacuum-packed into polythene bags and sent to the University of Queensland, School of Agriculture and Food Sciences, Australia, where they were maintained at -80°C until analysis (Daygon et al., 2017).

Sensory evaluation of the brown rice grain

Seven panelists (7) from the Nutrition and Bioanalytical lab, National Crops Resources Research Institute, Uganda were trained in a 2 h session for 3 days prior to the grain aroma evaluation. Forty grains of each genotype were soaked in 10 ml of 1.7% KOH solution at room temperature in a covered glass Petri-dish for 30 min following the method of Golam et al. (2010). Thereafter, the grain samples were coded and randomized for aroma evaluation immediately after removing the Petri-dish cover by using short sniffs. Four-point category scales (1 = no aroma, 2 = slight aroma, 3 = moderate aroma and 4 = high aroma) were used to measure the intensity of grain aroma.

*Corresponding author. E-mail: d.ocan@gu.ac.ug.

Table 1. Information on the 28 selected rice genotypes.

Genotype	Crosses	Pedigree	Origin	Aroma status
AGRA 41	O. sativa x O. sativa	AGRA-CRI-UPL-3-4	AfricaRice, Benin	Unknown
AGRA 55	O. sativa x O. sativa	AGRA-CRI-UPL-4-4	CRI, Ghana	Unknown
Namche 2	O. sativa japonica x (O. sativa japonica x O. glaberrima)	NM7-8-2-B-P-11-6	NaCRRI Uganda	Nonaromatic
Namche 5	O. sativa x O. sativa	NM7-27-1- B-P-77-6	NaCRRI Uganda	Nonaromatic
Namche 6	O. sativa x O. sativa	NM7-5-2- B-P-79-7	NaCRRI Uganda	Nonaromatic
Komboka	O. sativa x O. sativa	IR 05N 221	IRRI, Philippines	Aromatic
Supa 3	O. sativa x O. sativa	IR 97011-7-7-3-1-B	IRRI, Philippines	Aromatic
Supa 5	O. sativa x O. sativa	-	IRRI, Philippines	Aromatic
Supa 6	O. sativa x O. sativa	IR 9712-4-1-2-1-1	IRRI, Philippines	Aromatic
MET 3	O. sativa x O. barthi	ARTT35-114-1-6N-2	AfricaRice, Nigeria	Unknown
MET 4	O. sativa x O. barthi	ART34-146-1-8N-1	AfricaRice, Nigeria	Unknown
MET 6	O. sativa x O. barthi	ART35-49-1-4N-1	AfricaRice, Nigeria	Unknown
MET 12	O. sativa x O. barthi	ART34-88-1-2-B-1	AfricaRice, Nigeria	Unknown
MET 13	O. sativa x O. barthi	ART34-113-3-2-B-1	AfricaRice, Nigeria	Unknown
MET 14	O. sativa x O. barthi	ART34-256-3-1-B-2	AfricaRice, Nigeria	Unknown
MET 16	O. sativa x O. barthi	ART35-272-1-2-B-1	AfricaRice, Nigeria	Unknown
MET 33	O. sativa x O. barthi	ART27-58-6-2-1-1-3-1	AfricaRice, Nigeria	Unknown
MET 40	O. sativa x O. barthi	-	AfricaRice, Nigeria	Unknown
ART 4	O. sativa x O. sativa	-	AfricaRice, Nigeria	Unknown
ART 7	O. sativa x O. sativa	-	AfricaRice, Nigeria	Unknown
ART 10	O. sativa x O. sativa	-	AfricaRice, Nigeria	Unknown
Namche 4	O. sativa x O. sativa	ART3-11L1P1-B-B-2	AfricaRice, Nigeria	Nonaromatic
Sande TXD 306	O. sativa x O. barthi	O. barthi interspecific lines	AfricaRice, Nigeria	Unknown
Supa 1052	O. sativa x O. sativa	-	AfricaRice, Nigeria	Aromatic
1190	O.sativa japonica x O. glaberima	-	AfricaRice, Nigeria	Unknown
Nerica 4	O.sativa japonica x O. glaberima	WAB 450-1-B-P-91-HB	AfricaRice, Côte d'Ivoire	Nonaromatic
E 20	O. sativa x (O.sativa x O. glaberima)	IRAT 325/WAB 365-B-1H1-HB	NaCRRI, Uganda	Nonaromatic
Yasmin aromatic	O. sativa x O. sativa japonica	-	Egypt	Aromatic

Source: Extracted from Kanaabi et al. (2018).

Assessment of rice grain 2-acetyl-1-pyrroline levels

Assessment of 2AP concentration was performed in the Plant and Food Metabolomics laboratory, School of Agriculture and Food Sciences, The University of Queensland, Australia. Polished rice samples were ground cryogenically using a TissueLyser (Qiagen, Hilden, Germany) and 1 g of flour was placed in autosampler tubes, sealed and frozen at -80°C until analysis. The samples were prepared in triplicate. Once ready for analysis, samples were left overnight at room temperature to equilibrate, and then volatile compounds in the headspace

of each sample were analysed by GC×GC-TOF-MS. The sealed tubes were then randomised and analysed in batches of 50. Blank samples were run before analysis of the experimental samples to equilibrate the machine and a quality control (QC) sample was placed at every 10th position in the sample queue. The samples were analysed following previously published method **(**Daygon et al., 2017). Briefly, the samples were heated to 80°C with agitation for 10 min on a CombiPal Autosampler (Agilent, CA, USA) to volatilise compounds. The headspace (1.5 ml) was collected using a 2.5 ml headspace syringe at 80°C and injected in splitless mode (Pegasus 4D GC×GC-TOF-MS Leco; St. Joseph, MI, USA). The temperature of the GC inlet and transfer line was maintained at 250°C. Separation was performed first on a primary column (Agilent DB-624UI midpolar, 30m x250µ x1.4µ; Agilent, CA, USA) and then on a secondary column which was a Stabilwax (polar, 0.9m x250µ x0.50 µ; Restek, Bellefon, USA). The primary column was initially set to 45°C for 1 min and then ramped at a rate of 10°C/min to 235°C. The secondary column and the modulator were set at 15 and 25°C higher than the primary column, respectively for the entire run. The modulation period was set at 2.5s, with 0.4s hot pulse time and 0.85s cool time between stages. The carrier gas (Helium) was maintained at a constant flow rate of 1 ml/min. Data was acquired using a TOF-MS after a 200s delay with an acquisition rate of 200 spectra/s. The MS scanned analytes within the mass range of 35 to 500 m/z. The ion source was held at 240°C.

Data pre-processing, alignment and noise correction were done using ChromaTof v4.50. Signal to noise ratio was set at 25. The absence of instrument drift and batch effects was verified using the QC samples and technical replicates. Identification of 2AP was done by comparison of retention time and electron ionization (EI) fragmentation patterns of the samples to an in-house mass spectral library created by running authentic analytical standards (Daygon et al., 2017). The relative amounts of 2AP were calculated by measuring the area under the curve of the 2AP peak.

Statistical data analyses

Data were subjected to analysis of variance (ANOVA) using R-statistical software (R Core Team, 2017, Version 3.4.1). Fisher's protected least significance difference (LSD) was used to separate means at 5% level of significance. Principal component analysis was performed using the grain chemical constituent data to plot and visualize the rice lines based on 2AP abundance and regression analysis conducted using MS Excel Software to determine the proportion of variation in sensory aroma accounted by the change in 2AP abundance. Multiple comparison of the mean concentration of 2AP in 26 rice lines against controls for nonaromatic (NERICA 4) and aromatic (Supa 5) was performed (Rafter et al., 2002). For pairwise comparisons, Dunnett's t-test was conducted to determine significant ($p<0.05$) differences between the lines (Dunnett, 1955).

RESULTS AND DISCUSSION

Concentration of 2-acetyl-1-pyrroline in the rice genotypes

Gas chromatography data revealed that the relative amount of 2AP varied from 0.000 to 0.3195 (Table 2).

In ascending order of 2AP, the rice genotypes MET 12, MET 13, Nerica 4, Supa 3, MET 14, E 20, Namche 6, Namche 5, Namche 2, ART 4, Namche 4, MET 40, 1190, ART 7, Supa 6, and MET 4 had significantly ($p<0.05$) lower relative percentage concentration of 2AP compared to other rice genotypes studied, with the exception of ART 10 and Sande. In this category, the lowest relative percentage amount of 2AP was 0.000 and the highest 0.0531 constituting the category of the lowest 2AP concentration and MET 12, MET 13, Nerica 4, and Supa 3 appeared not to contain any 2AP within their aroma profiles. The genotypes ART 10, Sande and Komboka had significantly ($p<0.05$) lower relative percentage concentration of 2AP compared to AGRA 41, MET 6, MET 33, AGRA 65 and MET 16. For this second category, the relative percentage concentration of 2AP varied from 0.0642 to 0.1333 with genotypes, Supa 1052 and Yasmin aromatic having significantly ($p<0.05$) higher relative percentage concentration of 2AP compared to MET 3, Supa 5 and all the other genotypes studied. The category of genotypes with highest 2AP concentration appeared to be distinct from the other genotypes. The GC data revealed substantial variations in the 2AP aroma constituent among different rice genotypes and within the broad categories of rice genotypes from similar geographical sources e.g. MET and Supa came from Nigeria and Philippines, respectively. Variations in the aromatic profile concentration of 2AP among cultivars were reported by Pachauri et al. (2010). Given that the studied 28 genotypes constitute the predominantly cultivated rice in the country, it is evident that the national breeding program has invested less in the development of rice aroma traits. Kanaabi et al., (2018) reported genotype 1190 to be aromatic based on individual grain assessment by chewing following the procedure of Dhulappanavar (1976). The results showed that genotypes 1190, Supa 6 and MET 4 were in moderate aromatic category. Similarly, among the rice genotypes within the moderate 2AP category, MET 16. AGRA 55, AGRA 41 and MET 6 have shown to be in elevated 2AP profile and aromatic category.

Considering the genotypes constituting the family category under AGRA, ART, MET, Namche, and Supa, the Namche family had in general genotypes with the lowest scores of relative percentage fraction of 2AP (Table 2). In contrast, generally, the Supa family of genotypes had the highest scores of the relative percentage fraction of 2AP. The other families of ART, MET and AGRA had scores in between the values for Namche and Supa families. Within the specific rice families, the largest degree of 2AP variation (minimum vs. maximum) was observed among the Supa genotypes and the narrowest degree of 2AP variation was observed among the AGRA and ART families. Considering variation within the Supa genotypes, Supa 1052 and Supa 5 contained high concentrations of 2AP, but Supa 3 genotype had non-detectable levels of 2AP. This finding is inconsistent with earlier reports from several workers who had advanced the idea that all Supa lines, including Supa 3 were aromatic (Kikuchi et al., 2015; Kanaabi et al., 2018. thus, the indiscriminate reliance on the Supa

Table 2. The amount of 2AP (relative percentage fraction) and the sensory aroma perception score of the hulled rice grains.

Genotype	Minimum 2AP%	Maximum 2AP%	Mean 2AP %	Sensory score
Agra 41	0.1109	0.1828	0.1520^{f} ±0.0370	2.86^{j} ±0.86
Agra 55	0.1464	0.1772	0.1595^{fg} ±0.0159	2.14^{efg} ±0.86
Namche 2	0.0000	0.0057	0.0031^{ab} ±0.0029	1.50^{abcd} ±0.94
Namche 5	0.0000	0.0052	0.0017^{a} ±0.0030	1.07^{ab} ±0.27
Namche 6	0.0000	0.0034	0.0011^{a} ±0.0020	1.14^{ab} ±0.36
Komboka	0.0617	0.1324	0.0957^{de} ±0.0354	2.21^{efgh} ±0.80
Supa 3	0.0000	0.0000	0.0000^{a}±0.0000	1.28^{abc} ±0.47
Supa 5	0.2260	0.2751	0.2445^{i} ±0.0268	2.79^{ij} ±0.70
Supa 6	0.0300	0.0548	0.0432^{bc} ±0.0125	1.71^{cde} ±0.99
MET 3	0.1907	0.2757	0.2323^{hi} ±0.0426	2.71^{hij} ±0.99
MET 4	0.0319	0.0726	0.0531^{c} ±0.0204	2.43^{ghij} ±0.65
MET 6	0.1048	0.2052	0.1389^{f}±0.0575	2.64^{ghij} ±0.74
MET 12	0.0000	0.0000	0.0000^{a} ±0.0000	2.57^{ghij} ±1.09
MET 13	0.0000	0.0000	0.0000^{a} ±0.0000	2.50^{ghij} ±0.65
MET 14	0.0000	0.0023	0.0008^{a} ±0.0013	2.43^{ghij} ±0.94
MET 16	0.1591	0.2257	0.1940^{gh} ±0.0334	2.93^{j} ±0.73
MET 33	0.1277	0.1673	0.1500^{f} ±0.0203	1.00^{a} ±0.00
MET 40	0.0000	0.0158	0.0053^{ab} ±0.0091	1.57^{bcd} ±0.76
ART 4	0.0000	0.0052	0.0033^{ab} ±0.0029	1.07^{ab} ±0.27
ART 7	0.0347	0.0433	0.0392^{abc} ±0.0043	1.36^{abcd} ±0.50
ART 10	0.0584	0.0672	0.0642^{cd} ±0.0050	1.50^{abcd} ±0.65
Namche 4	0.0000	0.0148	0.0049^{ab} ±0.0085	1.00^{a}±0.00
Sande TXD 306	0.1091	0.1748	0.1333^{cd} ±0.0361	1.86^{def} ±0.95
Supa 1052	0.2849	0.3482	0.3217^{j} ±0.0329	1.29^{abc} ±0.47
1190	0.0000	0.0199	0.0066^{ab} ±0.0115	1.00^{a} ±0.00
Nerica 4	0.0000	0.0000	0.0000^{a} ±0.0000	1.14^{ab} ±0.36
E 20	0.0000	0.0027	0.0009^{a} ±0.0015	1.14^{ab} ±0.36
Yasmin aromatic	0.2551	0.3662	0.3195^{j} ±0.0576	2.29^{fghi} ±0.83

genotypes could offer less desirable outcomes in a conventional breeding programme. The result indicated that the concentration levels of 2AP present in several popular rice genotypes in Uganda suggested that they are nonaromatic, which explains the efforts aimed at improving aroma in the Uganda genotypes lines using newly introduced exotic germplasm (MAAIF, 2012). As GC estimation of the rice aroma intensity was primarily on the levels of 2AP concentration, an important constituent among several other aroma compounds, a more robust aroma profile investigation within the 28 lines was conducted using trained sensory panelists.

Sensory evaluation scores of the trained panelists revealed that 1190, ART 10, ART 4, ART 7, E 20, MET 33, MET 40, Namche 2, Namche 4, Namche 5, Namche 6, Nerica 4 and Supa 3 had significantly ($p<0.05$) lower rice aroma intensity compared to the other rice genotypes (Table 2). The category with scores ranging from 1.00 to 1.57 was ranked lowest in regards to rice aroma intensity. The AGRA 55, Komboka, and Yasmin aromatic with scores ranging from 2.14 to 2.29, was ranked second in regards to rice aroma intensity. The third category constituted by AGRA 41 and MET 16 was not clearly differentiated from MET 3, MET 4, MET 6, MET 12, MET 13, MET 14 and Supa 5 which were rated aromatic. In spite of the subjective nature of sensory evaluation, both GC and sensory evaluation determinations established three broad groupings of the genotypes studied in relationship to grain aroma intensity indicating that the trained panels employed in sensory evaluation studies could be more objective than subjective in awarding scores (Chakraborty et al., 2016). Previous aroma evaluation study based on 3 trained panelist with rank (0=non aroma, 1=moderate aroma and 2=high aroma) indicated aromatic rice to score at least 0.67 (Hien et al., 2006). By converting a scale of 1 for nonaromatic to 0 for nonaromatic, the limit for nonaromatic rice of 1.57 reported in this study and 0.67 according to Hien et al. (2006) seems to be comparable. However, the sensory evaluation approach was observed to suffer from a relatively lower discriminatory power in assigning the genotypes lines into the three different categories. This

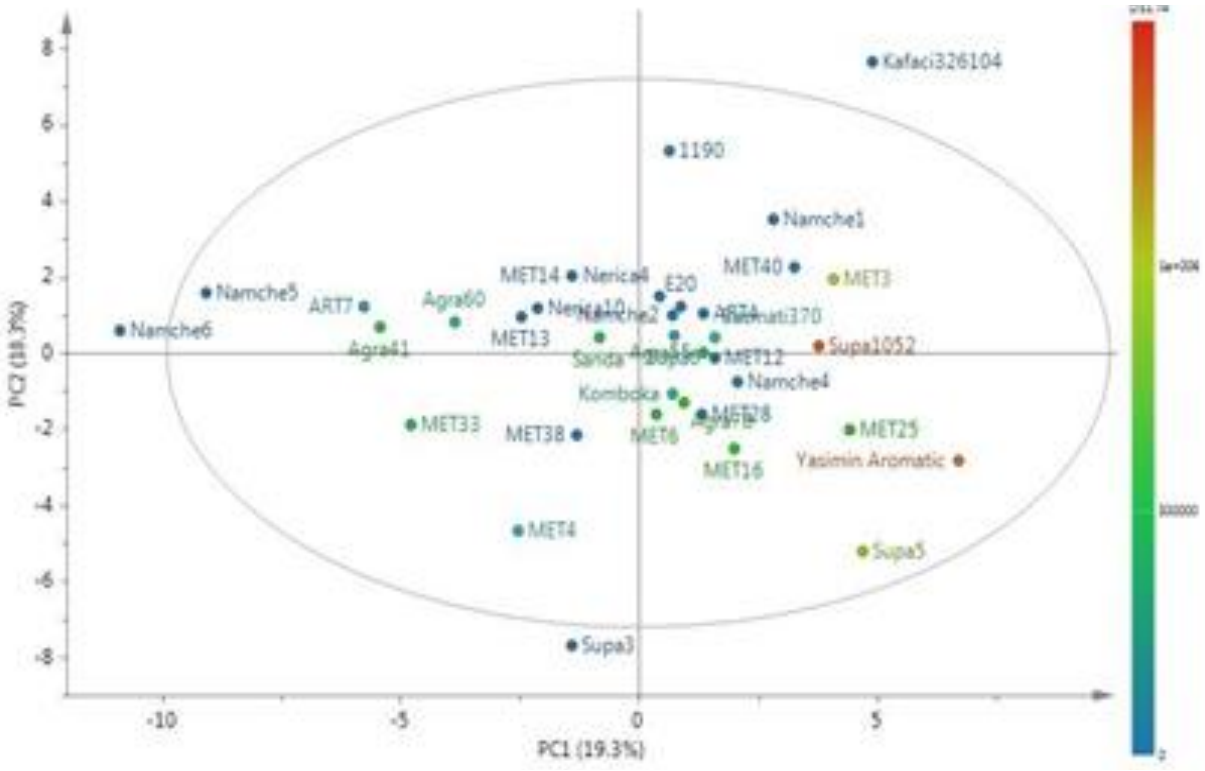

Figure 1. Scatter plot of two principal components of rice genotypes sorted based on the amount of 2-acetyl-1-pyrroline.

was probably due to the highly complex nature by which the humans olfactory sense perceive aroma in comparison to simplified chemical assays (Chambers and Kadri, 2013).

Among the genotypes in the AGRA, ART, MET, Namche, and Supa families, the Namche family had in general the lowest average score (1.18) of aroma intensity and the genotypes constituting AGRA family had the highest average score (2.5) of aroma intensity (Table 1). The genotypes constituting the family under ART, MET and Supa had intermediate average scores of aroma intensity ranked at 1.13, 2.31 and 1.77, respectively. Based on GC results, the largest variation in 2AP (minimum vs. maximum) was observed among the MET lines and the narrowest 2AP variation were exhibited among the genotypes in the Namche and ART families (Table 2). In principle, the result for ranking of genotypes with regard to aroma intensity within and between the families and categories using both techniques were in agreement. However, there were a few instances where the result from GC and sensory evaluation were not in agreement, for example Supa 1052 was rated highly aromatic (mean % = 0.3217) based on GC results and at the same time slightly aromatic (score=1.29) by sensory evaluation indicating the complex nature of aroma (Chamber et al., 2013) suggesting tendency of disagreement in assaying of the group with the widest variation in aroma between the GC and sensory evaluation methods (Chambers and Kadri, 2013). However, in regards to the family with the narrowest variation, fairly the two approaches were more in agreement implying that for genotypes with narrow 2AP scores, the sensory profile appeared to be more easily assayed compared to lines with wide concentration of 2AP. This could be due to the contribution of the other volatile organic compounds (VOC) causing complexity in aroma (Chambers and Kadri, 2013). This complexity account for the variability in 2AP based on two principal axes (Figure 1).

In general, both the GC and sensory evaluation methods tend to agree as indicated by the result from the simple regression analysis (Figure 2).

The results indicate that even when 2AP abundance is zero, the sensory score of up to 1.57 could be recorded by the panelist. This suggests the role of other compounds in rice aroma. The comparison of the two methods pointed out that percentage increase in 2AP of 2.95 would be required to cause a unit increase in aroma sensory score. Based on this study, the 2AP accounted for up to 19.8% of the total variation in sensory aroma perception in the rice grain. This further suggests the role of other compounds in determining the final grain rice aroma. This could be the reason as to why an aroma score of more than one (1) would be recorded in the absence of 2AP. However, the positive and significant ($p<0.05$) regression coefficient of 0.198 clearly show

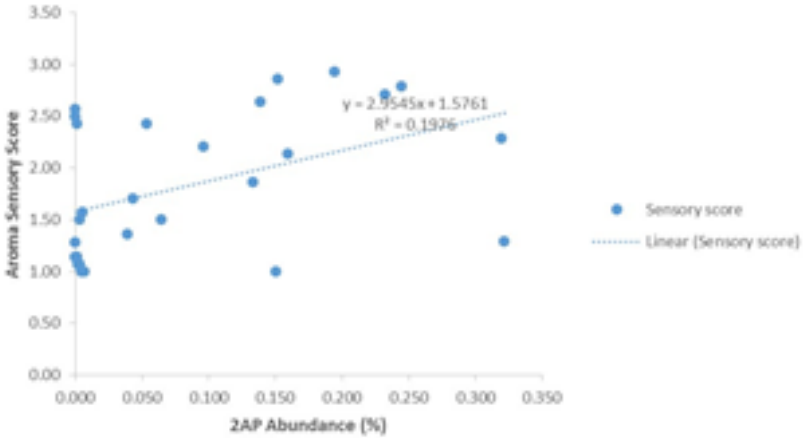

Figure 2. Trend line for correlation between 2AP amount and sensory score for aroma in rice.

correlation between sensory aroma and the percentage 2AP abundance in the rice grains.

Aroma classification of the genotypes lines

The result of classification of the genotypes based on the 2AP peak area of the different rice aroma profiles are presented in Table 3.

Nerica 4 (aromatic negative control), as compared to AGRA 41, AGRA 55, ART 10, Komboka, MET 3, MET 6, MET 16, MET 33, Sande, Supa 5, Supa 1052 and Yasmin aromatic which had significantly ($p<0.05$) higher 2AP mass ion abundance in their aroma profiles were classified as moderately aromatic, and MET 3, Supa 5, Supa 1052 and Yasmin aromatic were highly aromatic. Differentiation among the two aromatic sub-classifications was determined by the presence of a significant difference ($p<0.05$) against Nerica 4 with a simultaneous significant difference ($p<0.05$) against Supa 5 for moderately aromatic (Dunnet, 1955). For the highly aromatic sub-classification, a significant difference ($p<0.05$) against Nerica 4 with a simultaneous non-significant difference ($p>0.05$) against Supa 5 was the criteria used (Dunnet, 1955). With regards to comparisons against Supa 5 (aromatic positive control), all genotypes had significantly ($p<0.05$) lower 2AP mass ion abundance except MET 3, Supa 1052 and Yasmin aromatic. Thus, aroma based classification of Uganda genotypes using pairwise multiple means comparison also revealed the existence of three broad categories which were clearly defined suggesting that Nerica 4 is a reliable standard reference cultivar for denoting nonaromatic rice lines. Given that earlier workers classified Nerica 4 as aromatic (Kanaabi et al., 2018), the present study highlights the importance of simultaneous chemical assays (GC) with sensory evaluation in classification of genotypes. On the other hand, besides Supa 5 that was used for denoting aromatic genotypes in this study (Kanaabi et al., 2018), it is evident that MET 3, Supa 1052 and Yasmin aromatic can equally be used as aromatic positive controls. Within the genotypes in AGRA, ART, MET, Namche and Supa families, the AGRA and Namche families revealed the least degree of variation in the classification criteria using 2AP ion abundance (Table 3) and the MET family of rice lines had the highest degree of variation in the classification criteria using 2AP ion abundance.

Conclusion

Substantial variations in aroma intensity within and between the Uganda rice families were evident and the levels of aroma variation were strongly influenced by the type of rice family. The three categories of nonaromatic, moderately aromatic and highly aromatic were clearly differentiated. GC in tandem with complementary sensory evaluation indicated the highly complex nature of rice aroma as several rice lines were re-classified on the basis of a more comprehensive analysis. Thus, in developing a breeding strategy aimed at improving the aroma quantitative information about 2AP and sensory evaluation would be required in the future.

CONFLICT OF INTERESTS

The authors declare no conflict of interest regarding the publication of this paper.

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Table 3. Aroma classification of rice lines into aromatic classes.

Comparison with nonaromatic						Comparison with aromatic						
Genotype contrast	Observed difference ($m/z \times 10^6$)	Std. error observed difference ($m/z \times 10^6$)	t value	Pr(>|t|)	Sig.	Genotype contrast	Observed difference ($m/z \times 10^6$)	Std. error observed difference ($m/z \times 10^6$)	t value	Pr(>|t|)	Sig	Aroma class
Agra41 vs. Nerica4	0.5330	0.058	9.09	< 0.001	***	*Agra41 vs. Supa5*	-0.381	0.059	-6.49	< 0.001	***	MA
AGRA55 vs. Nerica4	0.5382	0.058	9.80	< 0.001	***	*AGRA55 vs. Supa5*	-0.340	0.059	-5.78	< 0.001	***	MA
NamChe2 vs. Nerica4	0.0099	0.058	0.17	1.0000	ns	*NamChe2 vs. Supa5*	-0.904	0.059	-15.41	< 0.001	***	NA
NamChe5 vs. Nerica4	0.0065	0.058	0.11	1.0000	ns	*NamChe5 vs. Supa5*	-0.907	0.059	-15.47	< 0.001	***	NA
NamChe6 vs. Nerica4	0.0048	0.058	0.08	1.0000	ns	*NamChe6 vs. Supa5*	-0.910	0.059	-15.58	< 0.001	***	NA
NamChe2 vs. Nerica4	0.0099	0.058	0.17	1.0000	ns	*NamChe2 vs. Supa5*	-0.904	0.059	-15.41	< 0.001	***	NA
Komboka vs. Nerica4	0.3526	0.058	6.01	< 0.001	***	*Komboka vs. Supa5*	-0.561	0.059	-9.57	< 0.001	***	MA
Supa3 vs. Nerica4	-0.0012	0.058	0.00	1.000	ns	*Supa3 vs. Supa5*	-0.913	0.059	-15.58	< 0.001	***	NA
Supa5 vs. Nerica4	0.9135	0.058	15.58	< 0.001	***	*Supa5 vs. Supa5*	-	-	-	-	ns	HA
Supa6 vs. Nerica4	0.1515	0.058	2.58	0.1871	ns	*Supa6 vs. Supa5*	-0.762	0.059	-13.00	< 0.001	***	NA
MET3 vs. Nerica4	0.9906	0.058	16.90	< 0.001	***	*MET3 vs. Supa5*	0.772	0.059	1.32	0.9647	ns	HA
MET4 vs. Nerica4	0.1853	0.058	3.16	0.483	ns	*MET4 vs. Supa5*	-0.728	0.059	-12.42	< 0.001	***	NA
MET6 vs. Nerica4	0.6083	0.058	10.37	< 0.001	***	*MET6 vs. Supa5*	-0.305	0.059	-5.20	< 0.001	***	MA
MET12= vs. Nerica4	-0.0003	0.058	0.00	1.000	ns	*MET12 vs. Supa5*	-0.913	0.059	-15.58	< 0.001	***	NA
MET13 vs. Nerica4	-0.0015	0.058	0.00	1.000	ns	*MET13 vs. Supa5*	-0.914	0.059	-15.58	< 0.001	***	NA
MET14 vs. Nerica4	0.0028	0.058	0.05	1.000	ns	*MET14 vs. Supa5*	-0.911	0.059	-15.53	< 0.001	***	NA
MET16 vs. Nerica4	0.6593	0.058	11.24	< 0.001	***	*MET16 vs. Supa5*	-0.254	0.059	-4.34	< 0.001	***	MA
MET33 vs. Nerica4	0.5139	0.058	8.76	< 0.001	***	*MET33 vs. Supa5*	-0.400	0.059	-6.812	< 0.001	***	MA
MET40 vs. Nerica4	0.0294	0.058	0.50	1.0000	ns	*MET40 vs. Supa5*	-0.884	0.059	-15.08	< 0.001	***	NA
ART4 vs. Nerica4	0.0107	0.058	0.18	1.0000	ns	*ART4 vs. Supa5*	-0.903	0.059	-15.40	< 0.001	***	NA
ART7 vs. Nerica4	0.1395	0.058	2.38	0.2802	ns	*ART7 vs. Supa5*	-0.774	0.059	-13.20	< 0.001	***	NA
ART10 vs. Nerica4	0.2367	0.058	4.04	<0.01	**	*ART10 vs. Supa5*	-0.677	0.059	-11.54	< 0.001	***	MA
Namche4 vs. Nerica4	0.0227	0.058	0.39	1.0000	ns	*Namche4 vs. Supa5*	-0.891	0.059	-15.19	< 0.001	***	NA
Sande TXD 306 vs. Nerica4	0.5382	0.058	9.18	< 0.001	***	*Sande TXD 306 vs. Supa5*	-0.375	0.059	-6.40	< 0.001	***	MA
Supa1052 vs. Nerica4	1.3791	0.058	23.52	< 0.001	***	*Supa1052 vs. Supa5*	0.4211	0.059	7.94	< 0.001	***	HA
1190 vs. Nerica4	0.0445	0.058	0.76	1.0000	ns	*1190 vs. Supa5*	-0.869	0.059	-14.82	< 0.001	***	NA
Nerica4 vs. Nerica4	-	-	-	-	ns	*Nerica4 vs. Supa5*	-0.914	0.059	-15.58	< 0.001	***	NA
E20 vs. Nerica4	0.0033	0.058	0.06	1.0000	ns	*E20 vs. Supa5*	-0.910	0.059	-15.52	< 0.001	***	NA
Yasmin Aromatic vs. Nerica4	1.3356	0.058	22.76	< 0.001	***	*Yasmin Aromatic vs. Supa5*	0.421	0.059	7.18	< 0.001	***	HA

NA = Nonaromatic; MA = Moderately aromatic; HA= Highly aromatic.

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Evaluation of aflatoxins levels and molecular identification of toxigenic molds in cereals and cereal-derived breakfast foods in Nigeria

Michael O. Odo, Fidelis Azi*, Ignatius C. Alaka and Veronica N. Nwobasi

Department of Food Science and Technology, Ebonyi State University, EBSU, Abakaliki, Ebonyi State, Nigeria.

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In the last decade, there have been an increase in safety concerns on the aflatoxins contents of cereals and cereal-derived food products in Nigeria. In the current study, evaluation of aflatoxins levels and molecular identification of toxigenic molds recovered from cereals and cereal-derived foods products in Nigeria were studied. Enzyme Link Immunosorbent Assay (ELISA) and high performance liquid chromatography (HPLC) methods were used for qualitative and quantitative aflatoxins evaluation, respectively. The result showed that aflatoxins were detected in 53 out of the 120 samples of the cereals and their products studied. The detected levels of aflatoxins in some of the samples were over the permissible (4 µg/kg) as recommended by relevant food regulatory authorities. Aflatoxins B_1, B_2, G_1, and G_2 were all detected in the sample, aflatoxin B_1 was the most predominant in the samples. *Aspergillus flavus* (AZ19), was isolated and identified as the major contaminating mold. Thus, findings of this research provide strong evidence that incidence of aflatoxins contamination of food crops still remain a major problem in Nigeria agricultural sector.

Key words: Aflatoxins, toxigenic molds, cereals, cereal-derived foods, *Aspergillus flavus*.

INTRODUCTION

Aflatoxins are toxic chemical compounds produced in foods and food products by *Aspergillus flavus* and *Aspergillus parasiticus*. These mycotoxins have been shown to induce both genotoxic and carcinogenic effects in humans (EFSA, 2007). Cereals and its products have been widely reported to be prone to contamination by potentially toxigenic fungi (Achaglinkame et al., 2017). However, incidences of aflatoxins contamination of cereals and associated food products are more prevalent in developing countries such as Africa compared to Europe (EFSA, 2013; Wagacha and Muthomi, 2008). The prevalence of aflatoxins contamination of cereals and cereals derived food products in Africa is due to improper post-harvest handling and/or storage in addition to the inability to control environmental factors (high temperature, high relative humidity and moisture content) that promotes the growth of the toxigenic mold and mycotoxins production (Gong et al., 2003; Beuchat, 2002; Burger et al., 2013). Studies have shown that high aflatoxins exposure in young children in Africa is due to feeding in contaminated cereals and cereal-food products either as complementary or breakfast food (Gong et al.,

*Corresponding author. E-mail: azifideel@yahoo.com or azi.fidelis@ebsu-edu.net. Tel: +2348067872449.

2003; Magoha et al., 2016; Achaglinkame et al., 2017). The maximum permissible levels of aflatoxins B1 and aflatoxins B2, G1 and G2 in all cereals and cereal-based human foods are 2 and 4 µg/kg, respectively as recommended by EFSA (2013). There are significant adverse carcinogenic potentials of exposure to aflatoxins over the permissible limits for long period. The most dangerous effects of AFs in human especially in children are the immune suppression effect, low birth weight, and growth impairement (Lombard, 2014; Wagacha et al., 2008). Whereas chronic exposure to aflatoxins have been shown to cause different kinds of cancer (Gong et al., 2016).

On the other, hand maize (*Zea mays*), millet (*Pennisetum glaucum*) and sorghum (*Sorghum bicolor*) are common foods in use in Nigeria. These cereals are very popular for their use in making "*pap*", quick breakfast beverages in Nigeria. *Pap* is a naturally fermented porridge that serves as both complementary weaning food for infant and breakfast food for adults in Nigeria and other African countries. Several studies have shown that due to improper post-harvest handling and bad storage conditions in Africa, these cereals are widely contaminated by potentially toxigenic fungi together with their toxins and these cereals must be subjected to examination before being used in making *pap* and breakfast beverages (Gong et al., 2003; Enyisi et al., 2015). Hence, the major setbacks to the utilization of these cereals in Africa are their susceptibility to aflatoxins contamination.

Furthermore, according to the European Commission all aflatoxins must be absent in agricultural products to be fit for human consumption (European Commission, 2006a). However, aflatoxins, in foods are stable in foods under different processing conditions including under cooking temperatures, hence difficult to eliminate (Magoha et al., 2016). Thus, the study by the Joint Expert Committee on Food Additives (JECFA) recommended that aflatoxins levels in foods and food products should be under permissible limit (Strosnider et al., 2006).

Moreover, EFSA (2011) reported that developing are the major countries exposed to aflatoxins in food commodities and hence appropriate preventive measures must be applied to reduce incidences of aflatoxins contamination.

Therefore, the purpose of this study is to evaluate aflatoxins contamination and molecular identification of toxigenic molds in cereal and cereal-derived breakfast food in Nigeria.

MATERIALS AND METHODS

Sample collection

One hundred and twenty (120) samples were collected and analyzed; twenty samples each of maize, millet and sorghum and the respective *pap* from sellers covering different markets in Abakaliki, Nigeria.

Isolation and identification of molds

The samples were first screened for the presence of viable mold spores using potato dextrose agar (PDA). The cereals and their products were cultured on PDA and incubated at room temperature of 25 to 28°C for 3 to 5 days. The recovered fungal cells were identified microscopically using morphological and microscopic features and were further identified by molecular biology to accurate species level using internally transcribed spacer (ITS) and partial calmodulin and transcriptional elongation factor (TEF) rDNA sequencing according to the method described by Azi et al., (2017).

Molecular identification of fungal isolate

The recovered mold isolates identified as *Aspergillus* species were further identified to species level at Centre for Agriculture and Biotechnology International (CABI), Microbial Identification Services (United Kingdom). ITS, partial calmodulin and TEF rDNA sequencing analyses were used for the identification of the isolates. All procedures were validated and processed in accordance with CABI's in-house method as documented in TPs 61-68 and TP70. Molecular assays were carried out using nucleic acid as a template. A proprietary formulation [microLYSISR-PLUS (MLP) Microzone, UK)] was subjected to the rapid heating and cooling of a thermal cycler, to lyse cells and release deoxyribonucleic acid (DNA). Following DNA extraction, polymerase chain reaction (PCR) was employed to amplify copies of the rDNA *in vitro*. The quality of the PCR product was assessed by undertaking gel electrophoresis. PCR purification step was carried out to remove unutilized dNTPs, primers, polymerase and other PCR mixture compounds and obtained a highly purified DNA template for sequencing. Sequencing reactions were undertaken using BigDyeR Terminator v3.1 kit from Applied Biosystems (Life Technologies, UK) which utilizes fluorescent labeling of the chain terminator ddNTPs, to permit sequencing.

Removal of excess unincorporated dye terminators was carried out to ensure problem-free electrophoresis of fluorescently labeled sequencing reaction products on the capillary array AB 3130 Genetic Analyzer (DS1) DyeEx™ 2.0 (Qiagen, UK). Modules containing prehydrated gel-filtration resin were optimized for clean-up of sequencing reactions containing BigDyeR terminators. Dye removal was followed by suspension of the purified products in highly deionized formamide Hi-Di™ (Life Technologies, UK) to prevent rapid sample evaporation and secondary structure formation. Samples were loaded onto the AB 3130 Genetic Analyzer and sequencing were undertaken to determine the order of the nucleotide bases, adenine, guanine, cytosine, and thymine in the DNA oligonucleotide. Following sequencing, identifications were undertaken by comparing the sequence obtained with those available in the European Molecular Biology Laboratory (EMBL) via the European Bioinformatics Institute (EBI).

Qualitative ELISA aflatoxins detection in the cereals and cereal-derived products

Detection of total aflatoxins in cereals and their *pap* samples was done by Enzyme Link Immunosorbent Assay (ELISA) method as described by Azi et al., (2017). Briefly, the samples were first ground into fine powder and aflatoxins content was extracted by addition of tween-ethanol (25 ml) to 5 g of each sample and mixed properly. Then the sample solution was centrifuged for 3 min at 250 rpm and filtered with Whatman No. 1 filter paper. Aflatoxin conjugate (200 µl) was dropped in a clean mixing wall and 100 µl of the sample analyte were added. The mixture of the aflatoxin conjugate and the sample was then transferred into antibody incubated micro-walls and incubated under dark condition at room

Table 1. Concentrations of total aflatoxins in the cereals and their *pap*.

Cereals/Cereal products	Number sample/positive	Aflatoxins concentrations (µg/kg)		Average value (µg/kg)
		Below limit	Above limit	
Maize	20/13	9 (4.3-5.7)	4 (11.3 -14.7)	9.52
MP	20/8	4 (4.6-6.4)	4 (10.2-12.3)	8.64
Millet	20/11	3 (1.8-3.8)	8 (5.7-7.8)	6.51
MiP	20/9	5 (2.1-3.3)	4 (4.1-5.6)	3.73
Sorghum	20/9	7 (1.9-3.1)	2 (4.9-5.2)	2.85
SP	20/3	2 (2.5-3.7)	1 (4.0)	2.31

MP= Maize *pap*, MiP=millet *pap*, SP=sorghum *pap.*

temperature for 15 min which allowed antibody/antigen reaction to take place. At the end of the incubation period, the solution was then washed off 5 times using deionized water and then 100 µl of the substrate were added and allowed to stand for 5 min. Finally, the solution was subjected for reading the aflatoxins content using ELISA machine.

Quantitative HPLC analysis of aflatoxins B_1, B_2, G_1 and G_2

The analyses of samples for aflatoxins B1, B2, G1, and G2 for all samples were also performed using Waters Alliance 2695 HPLC System, Column (C18, 4.6 × 150 mm) equipped with a 2475 Scanning Fluorescence detector. In this method, the mobile phase was water: methanol: acetonitrile (60:25:15 v/v/v) with a flow rate of 1.0 mL/min and the column temperature was 28°C and injection volume 50 µl. The fluorescence detector was operated at an excitation wavelength of 365 nm and an emission wavelength of 455 nm. The fluorescence of G1 and B1 was enhanced using electrochemically generated bromine. The analog out was sample energy while the scaling factor was 10 with the filter as digital (hamming) at 20 s. Post-column electrochemical derivatization was performed in order to enable detection of B_1 and G_1 aflatoxins. Detection was carried out at 220 nm. The resolution peaks were recorded on the HPLC chart according to the retention time of each aflatoxin. The concentrations of the aflatoxins were quantified from standard curves.

RESULTS AND DISCUSSION

Molecular identification of isolate

The natural adverse environmental conditions together with improper long-term storage are the major factors responsible for the presence of *Aspergillus* in food products (Campos et al., 2017; Burger et al., 2013). The *Aspergillus* contamination of cereals at different stages of the production chain such as pre-harvest, harvest, and post-harvest handling in Africa have been widely reported (Smith et al., 2016; Atanda et al., 2011). The findings of the current study revealed that the cereals and cereal-based foods studied were contaminated by *Aspergillus* spp. (Figure 1). While molecular identification showed that the cereals and their *pap* were mostly contaminated by toxigenic *A. flavus* (AZ19) strains which produce AfB and AfG (Luttfullah and Hussain, 2011). These cereals and their products are widely and frequently consumed in Nigeria as well as other African countries as basic staple foods. The presence of these potent toxigenic molds in these cereals and its product (*pap*) results in great health risk for a large number of human populations (children and adult) in Nigeria. This contamination could be as a result of bad storage and unhygienic processing facilities which might have provided optimum good conditions for the growth and proliferation of these fungi. The growth and toxin production by *Aspergillus* require about 13% moisture, relative humidity of 65% (water activity, a_w, of 0.65) (Medina et al., 2014; Achaglinkame et al., 2017). These factors together with improper drying might have predisposed the contamination of the cereals with *Aspergillus* and progressive proliferation.

Total aflatoxins in the cereals and their *pap*

The result of total aflatoxins (sum of B_1, B_2, G_1, G_2) in the cereals and their *pap* is shown in Table 1. Total aflatoxins levels in some of the cereals and their *pap* were above the maximum permissible limit as stipulated by relevant international regulatory authorities (4 µg/kg for cereals and cereal-derived products except maize for which maximum levels are 10 µg/kg) (European Commission, 2006b; EFSA, 2013). Currently, the maize sample was highly contaminated by fungi (13 out of 20 samples) followed by the millet sample while the least contamination was recovered from sorghum samples. This is in line with the findings of EFSA (2013) in which maize was the most contaminated cereals studied. On the other hand, fungal contamination of the *pap* produced from millet samples was more than those produced from sorghum. Among the contaminated maize samples, 4 samples were beyond safe limit of 10 µg/kg stipulated by European Union (EU) regulations for total aflatoxins whereas nine samples (2.3 to 5.7 µg/kg) had aflatoxins below the safe limit. Four samples of the maize *pap* also had unacceptable levels of total aflatoxins contaminations. The millet had eleven (11) contaminated samples out of the samples evaluated. While three of the samples had below maximum acceptable limit of aflatoxins

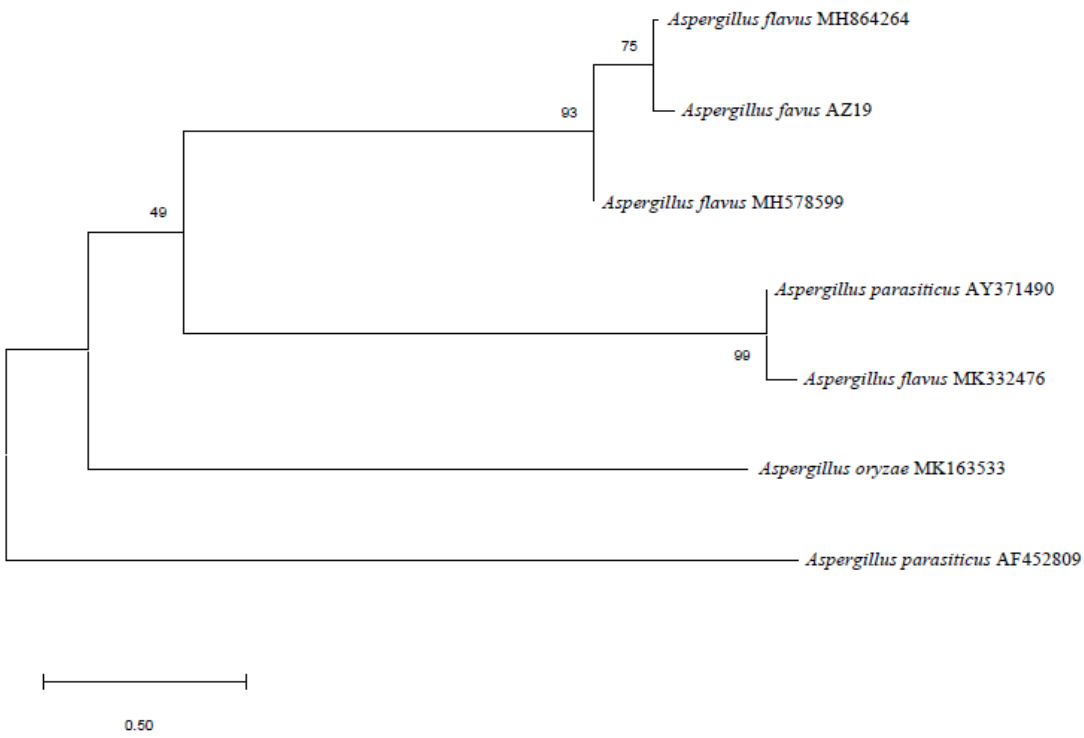

Figure 1. Phylogenic tree of the isolated *Aspergillus flavus* (AZ19).

contamination, eight samples had aflatoxins levels beyond the acceptable limit of 4 µg/kg according to European regulations. The millet *pap* also had nine samples contaminated above the limit of detection (LOD) of 1 µg/kg and four out of the nine samples were above the acceptable limit. Sorghum and its *pap* were the least contaminated of the cereals evaluated. It was only two out of the nine aflatoxins contaminated sorghum samples that had above limit contamination while one out of the three contaminated sorghum pap had above the acceptable limit of aflatoxins contamination. Mean occurrence values for maize and its *pap* were 9.54 µg/kg and 8.64, respectively. The millets and its *pap* were 6.51 and 3.73 µg/kg while the sorghum and its *pap* had the least average values of 2.85 and 2.31 µg/kg, respectively.

In general, 53 (44.16%) out of the 120 samples of cereals and their *pap* evaluated had values above 1 µg/kg LOD for the sum of aflatoxins B_1, B_2, G_1, and G_2. Fourth three percent (43.39%) samples had total aflatoxins contaminations above the permissible limit according to the regulations of the European Commission.

Thus, cereals and their pap with aflatoxins contamination above the permissible limit portend significant health risk for human exposure to the toxicants (European Commission, 2006a; EFSA, 2013). The high level of aflatoxins contamination of these cereals and their products might have been as a result of poor post-harvest handling/storage facilities. Most of the storage and processing structures commonly used by farmers/ local cereal processors in Nigeria as well as other Africa countries are traditional hence predisposes the cereals to *Aspergillus* contamination and subsequent aflatoxins production (Atanda et al., 2011). Environmental factors such as temperature and high relative humidity might have also played a significant role in increasing the levels of aflatoxins contaminations of these cereals. Temperature above 20°C has been reported to enhance the growth of *Aspergillus* while aflatoxins production is optimum at the temperature between 25 and 37°C (Smith et al., 2016; Strosnider et al., 2006). These temperatures are the ambient temperature in Nigeria and might have high relative humidity to be responsible for the rapid growth and multiplication of the toxigenic molds.

The variation in the prevalence and concentrations of aflatoxins in the different cereals and their product could be attributed to the differences in environmental factors during storage and processing as well as nutrient composition of the cereals. The level and rate of production of aflatoxins in food substrates have been reported to be influenced by available nutrients (Williams et al., 2003; Achaglinkame et al., 2017). Foods that have high concentrations of sucrose, maltose, and glucose have been noted to be more vulnerable to aflatoxins contamination (Magoha et al., 2016; Smith et al., 2016; Achaglinkame et al., 2017). Thus, the levels of aflatoxins and prevalence in the different cereals and their *pap* could also be partly attributed to the varying concentrations of the sugars present in the cereals.

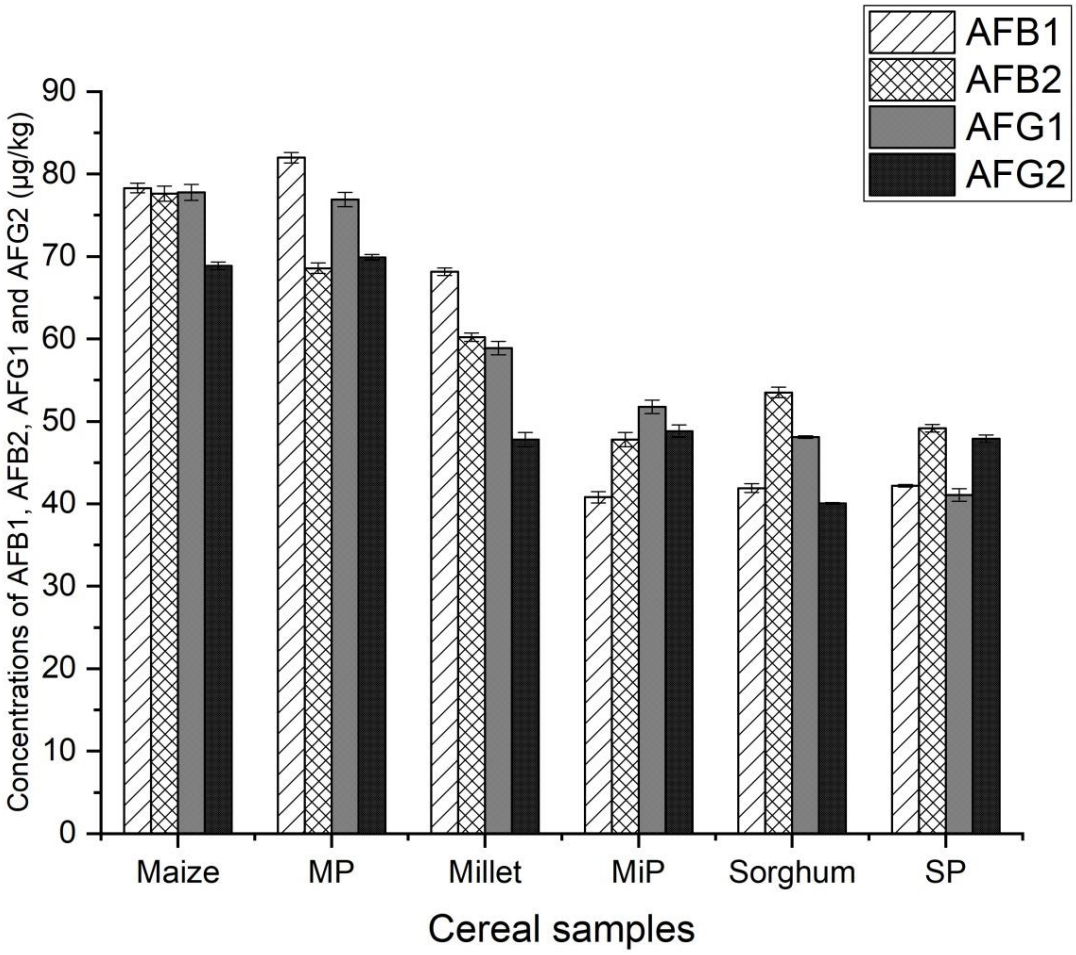

Figure 2. Average concentrations of aflatoxins B1, B2, G1 and G2 in the cereals and their *pap*.

Aflatoxins B1, B2, G2 and G2 contaminations in the cereals and their *pap*

Results of 120 cereal samples and their pap samples are summarized (Figure 2). The result showed that aflatoxins B_1, B_2, G_1, and G_2 were significantly detected in all the cereals analyzed with reference aflatoxins B1 being the most detected form in all the cereals and their products. Aflatoxin B1 is produced by *A. flavus* hence Figure 1 shows the evidence for its abundance in the cereals and their *pap* studied in this research.

Aflatoxin B1 is the most toxic and carcinogenic type than other aflatoxins ($B_1 > G_1 > B_2 > G_2$) and has been implicated in the etiology of hepatocarcinoma as well as tumors of the lungs and kidneys (Shephard, 2008). Chronic exposure increases the risk of developing liver and gallbladder cancer (Gong et al., 2016). Aflatoxins interact with the basic metabolic pathways of the human cells and disrupt key enzymatic processes including those involved in carbohydrate and lipid metabolism as well as protein synthesis (Shephard, 2008; Quist et al., 2000). It also induces changes in insulin-like growth protein factor, hence resulting in inhibition of mineral bioavailability especially in children and young adult (Sowley, 2016).

The impact of aflatoxins exposure is often most severe among children and pregnant mothers. Based on the exposure level, the effect can range from low birth weight to growth impairment, immunosuppression and mental retardation (Gong et al., 2004). In addition, Gong et al. (2016) reported that infants and young children who had the toxin's exposure above tolerable daily limit were shorter and under-weight. It was also recorded that in some cases it caused various kinds of cancer and resultant deaths depending on the type, period and amount of exposure (Lombard, 2014). From the finding of this study, cereals and their *pap* represent significant health risk considering that they are widely used as breakfast and weaning food in Nigeria. Similar studies carried out on cereals cultivated and consumed in other Africa countries such as Ghana, Kenya, Tanzania, Mali, and Benin show similar results of high aflatoxins

contamination with its attendant health risk (Magoha et al., 2016; Wagacha and Muthomi, 2008; Gong et al., 2004; Achaglinkame et al., 2017).

Conclusion

The findings of this study detected aflatoxins contamination of food crops especially cereals in Africa particularly in Nigeria. Unfortunately, daily consumption of these contaminated cereals and their products as either breakfast food or weaning food by infants and children still remains a reality in Nigeria as well as other African countries. Therefore, the present study warrants that urgent step be taken to raise more awareness on the incidences of mycotoxins contamination of our food crops. Furthermore, there is need for regular examination of cereals and their products for aflatoxins contamination.

It is therefore imperative that aflatoxins surveillance research findings such as the one presented in this study should form the basis for taking different prevention approach especially in the formulation/vigorous implementation of already existing national food policy that will help to reduce, if not eradicate, aflatoxins contamination of Nigeria agricultural produce. Critical among the strategies to be adopted should be a public campaign on the impact of aflatoxins on our health and the best control strategies to be adopted by all relevant stakeholders in the Nigerian agricultural sector.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Whole-genome optical mapping: Improving assembly of *Macrophomina phaseolina* MS6 through spanning of twelve blunt end chromosomes by obviating all errors and misassembles

Quazi Md. Mosaddeque Hossen[1]*, Md. Shahidul Islam[1], Emdadul Mannan Emdad[1], Md. Samiul Haque[1], Md. Monjurul Alam[1] and Maqsudul Alam[1,2]

[1]Basic and Applied Research on Jute (BARJ) Project, Bangladesh Jute Research Institute, Dhaka, Bangladesh.
[2]Advanced Studies in Genomics, Proteomics and Bioinformatics, University of Hawaii, Honolulu, Hawaii 96822, USA.

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Deciphering genetic information through next-generation sequencing (NGS) is considered as the basic platform to unveil in details of an organism. However, as it produces short reads that lead to difficulties in assembly, we generated long scaffold-based optical mapping (OM) data of previously sequenced devastating fungus, *Macrophomina phaseolina* MS6. In the process, *Kpn*I identified as the most effective restriction endonuclease among tested 13, used to digest high molecular weight (HMW) DNA that generated 270,343 genomic DNA molecules size in more than 250 kb. The molecules were assembled and constructed 12 super-scaffolds (terminated with telomeric blunt-ends and denoted as chromosomes) that were aligned with NGS generated 17 (out of 88 reduced from 94) reference scaffolds. The state-of-the-art technology revealed concordances and different discordances viz., inversions, low-quality assembly, gaps, overlaps followed to correct the NGS misassembles. Based on the results, OM generated improved and validated assembly advance our understanding of the chromosome evolution of fungi. This furnished data might be considered as valuable resources to accelerate the precise planning for the protection of *M. phaseolina* MS6 infected sequenced crops through developing the cross-talk phenomenon between the host and pathogen.

Key words: *Macrophomina phaseolina* MS-6, optical mapping, assembly improvement, assembly validation.

INTRODUCTION

Genome sequencing is the process of determining the full DNA sequences of living organisms including its chromosomal, mitochondrial and chloroplast DNA. Although DNA sequencing seemed not to be an easier and faster process, the rapid development of different pipelines and techniques made the whole genome sequences simple over the last few years (Koboldt et al., 2013). However, NGS produces a large number of short reads restraints the *de novo* assembly due to repeat or complex region of genome that suffers extensive misassembles and comprise gaps (Pendleton et al., 2015; Ganapathy et al., 2014; Ruperao et

*Corresponding author. E-mail: mosaddeque@jutegenome.org.

al., 2014). Therefore, the demand of introducing a new technique was a must to minimize these errors in terms of whole-genome sequencing.

Whole-genome optical mapping, the cutting edge technology offered for resolving the issues through estimating the gap length between the scaffolds and merges them into much longer sequences without introducing new bases (Ghurye and Pop, 2019; Kremer et al., 2017; Zhou et al., 2009; Aston et al., 1999; Samad et al., 1995). It also provides a valuable template for *de novo* genomic sequence assembly where large structural variations in the genome can accurately be detected and quantified (Long et al., 2018; Mak et al., 2016; Shukla et al., 2009; Teague et al., 2010). Furthermore, OM is capable of producing high-resolution, ordered, high-throughput genomic map data that gives information about the structure of a genome (Mukherjee et al., 2018; Schwartz et al., 1993). Though initially, it has been used to construct whole-genome restriction maps of bacteria, parasites, and fungi (Lai et al., 1999; Lim et al., 2001; Lin et al., 1999), recently it has been used for scaffolding contigs and for assembly validation of large-scale sequencing projects including maize, goat and *Amborella* genomes (Chamala et al., 2013; Udall and Dawe, 2018; Dong et al., 2013).

In Bangladesh, Jute (*Corchorus* species) is the most important cash crop considered the second foreign earning resources of the country (BBS, 2011). However, this crop is affected by several pathogens and diseases throughout its growing season and causing severe yield losses (Mamun et al., 2016). Among different agents *Macrophomina phaseolina* MS6, an ascomycetous, necrotrophic, soil-borne fungi, can solely reduce its yield up to 30% (Islam et al., 2012). This pathogen has more than 500 hosts (Lodha and Mawar, 2019; Khan et al., 2017; Islam et al., 2012) including major crops like cotton (Aly et al., 2007), jute (Meena et al., 2015; De et al., 1992), groundnut (Islam et al., 2012), maize (Biemond et al., 2013), sorghum (Su et al., 2001), millet (Lodha and Mawar, 2019), potato (Abbas et al., 2013), sesame (Dinakaran and Mohammed, 2001), soybean (Wyllie, 1993), beans (Mayek-Pérez et al., 2001), sunflower (Khan, 2007), sweet potato (Da Silva and Clark, 2013), tomato (Hyder et al., 2018), and tobacco (Wyllie, 1998). It outbreaks as stem rot (Majumder et al., 2018), seedling blight (Lu et al., 2015), charcoal rot (Majumder et al., 2018), dry root rot (Živanov et al., 2019), wilt (Piperkova et al., 2016), leaf blight (Mahadevakumar and Janardhana, 2016), pre and post-emergence damping-off (Hai et al., 2017), root and stem rot of softwood forest and fruit trees and also in weed species (Singh et al., 1990; McCain and Scharpf, 1989). This fungus forms microsclerotia in the soil and survives up to 15 years without attacking hosts (Kaur et al., 2012). It can also live in extreme environmental conditions like high temperature (30-35°C), low soil moisture, diverse pH, wide-ranging salt state and drought situation (Mengistu et al., 2011).

Considering all aforementioned consequences especially in jute, its genome was sequenced previously to have its mechanisms of attacking crops (Accession: AHHD00000000). But as described earlier, the bottlenecks that suffered the whole genome sequencing project, we addressed optical mapping to furnish and improve the genome. The furnished assembly, considered as valuable resources that might be used to develop a logical strategy for controlling the pathogen by unveiling the host-pathogen interaction within all sequenced crops that are infected by the pathogen.

MATERIALS AND METHODS

Preparation of working sample from *M. phaseolina* MS6

The strain *M. phaseolina* MS6 was taken from a stem rot infected jute plant (*Corchorus capsularis* L). The pathogen was cultured and purified on Potato Dextrose (PD) media maintaining 30°C for 72 h in dark conditions. The grey-brown mycelia were collected and washed with physiological buffer (Na_2HPO_4, pH 7.0: $NaH_2PO_4.H_2O$, pH <7.0) followed by drying under laminar flow Hood (Islam et al., 2012).

Extraction of megabase size DNA

Spheroplasting

Two grams of mycelia was ground into fine powder in liquid nitrogen with a mortar and pestle and immediately transferred into an ice-cold 1000 ml beaker containing 800 to 1000 ml ice-cold 1x Homogenize Buffer (HB) (0.1 M Tris, 0.8 M KCl, O.1 M EDTA, 10 mM Spermidine, 10 mM Spermine) with 0.15% beta-mercaptoethanol and 0.5% Triton X-l00. The contents were swirled gently for 10 minutes on ice and filtered by two layers of cheesecloth followed to one layer of Miracloth (Sigma-Aldrich, USA). The homogenate was taken into a centrifuge to have the pellet with a fixed-angle rotor at 1,800 g at 4°C for 20 minutes. The supernatant was discarded and approximately 1 ml of ice-cold Wash Buffer (WB) (1x HB, 20% TritonX-100, 0.15% beta-mercaptoethanol) was added to each tube. The pellet was resuspended gently with a small paintbrush soaked in ice-cold wash buffer. The nuclei were pelleted by centrifugation at 1,800 g at 4°C for 15 minutes in a swinging bucket centrifuge. The pellets were washed additional three times by resuspending in washing buffer using a paintbrush followed by centrifugation at 1,800 g at 4°C for 15 minutes. The pelleted nuclei were resuspended again in a small amount (1 ml) of 1x HB without beta-mercaptoethanol followed by counting the nuclei (approx. 5×10^7 nuclei/ml), under the contrast phase microscope with addition of the 1x HB without beta-mercaptoethanol and stored on ice.

Embedding cells

Low-melting-point (LMP) agarose (1%) was prepared in 1x HB without beta-mercaptoethanol and Triton X-100 followed by cooling down to 45°C and maintained in a 45°C water bath before use. The nuclei were pre-warmed to 45°C in a water bath (5 minutes) and mixed with an equal volume of the pre-warmed 1% LMP agarose in 1x HB without beta-mercaptoethanol and Triton X-100 using a cut-off pipette tip. The mixture was aliquoted into ice-cold plug molds on ice with the same pipette tip at 100 ml per plug.

Lysis

The solidified gel was sliced into pieces and incubated in 50 ml of digestion buffer (0.5 M EDTA, 7.5% β-mercaptoethanol) at 37°C for overnight. The buffer was replaced with NDSK buffer (0.5 M EDTA, 1% (w/v) N-lauroylsarcosine, 1 mg/ml proteinase K) for downstream work.

Plugs washing

The plugs were placed in a new 50 ml conical tube and added 45 ml of 1X TE buffer. The conical tube was capped with a clean green sieve and a regular cap and rock on a platform rocker at low speed for 1 h. 1X TE buffer was decanted from the conical tube and added fresh 1X TE buffer followed by rocking on a platform rocker at low speed for another 1 h. It was repeated for three times.

Melting plugs

The plugs were transferred into a sterile petri-dish and cut using a sterile scalpel in half and transferred to a separate 2.0 ml microcentrifuge tube. The microcentrifuge tubes were taken into a heat block at previously maintained 70°C for 7 minutes followed by pipetting 50 µl of the pre-warmed β-Agarose-TE solution (mixing 48 µl of 1X TE with 2 µl of 1 U/µl β-Agarose). The tube was incubated at 42°C heat block for overnight. Loading buffer was added to the DNA solution and stored at 4°C.

Restriction enzyme selection

The Enzyme.pl script (In-house script) was used to select optimal restriction endonuclease that generates restriction fragment statistics for different restriction enzymes. The optimal restriction enzyme was selected using this script by considering average fragment size (kb), fragment greater than 100 kb, maximum fragment size and the highest percentage of average fragment size underlie within 5 to 20 kb size fragments.

MapCard setup and data collection

The high molecular weight DNA was placed on optical chips to make them linear. This immobilized DNA was digested randomly with a restriction endonuclease and subsequently stained with jojo-1TM dye (Life Technologies Corporation, USA) for image capturing and fragment size measurement. The mapset (total data sets generated from all runs) was put together for assembly by using the Argus system embedded Gentig map assembler to create a consensus optical map. The mapsets were considered for assembly after filtering them following minimum molecule length (>150 kb) with minimum fragments per molecule (>12) and minimum molecule quality score (0.2).

Optical mapping assembly

The data from each MapCard were combined for the final assembly. In the assembly process, the filtered mapsets were taken for aligning them to form contigs by overlapping and keeping some extending fragments for resolving both side blunt ends (telomeric end protection). The final restriction maps were obtained by fulfilling criteria like coverage, depth, genome complexity of primary and final draft contigs. Different errors like low occurrence, low depth, and potential misassembles, as well as potential problems like false cut, missing cut, and false fragments were obviated using find hits techniques or removing the errors through Argus optical mapping embedded Gentig software packages. Assembly was conducted also with the removal of default circularization parameters. Partial assembly results were saved when 12 contigs became apparent by having >50 molecules each. Contigs were split off and reassembled against the original mapset individually using the "Find Hits" feature. Contigs were considered "finished" when no additional molecules were added by subsequent reassembles. Chromosome ends that were not blunt were visually inspected and any questionable molecule was removed from the final map (Figure 1).

Alignment by MapSolver

MapSolver software uses a dynamic programming algorithm to find the optimal placement location of each supplied sequence scaffolds in the Optical Map. The algorithm applies user-provided settings toward generating local alignments between each scaffold and the optical map. Scaffolds are aligned in both forward and reverse directions. MapSolver determines an alignment score for each comparison, where a higher score implies greater confidence in the alignment. Alignments with scores that meet or exceed the minimum score for local parameters are evaluated for placement. The number of aligned fragments must also meet or exceed the number specified by the Minimum aligned fragments parameter. In this study, default parameters were used.

RESULTS

Restriction endonuclease selection

A total of 13 restriction enzymes were evaluated to select the most efficient and suitable restriction endonuclease to consider different parameters including average and maximum size of DNA fragment and number of fragments. The effects of different restriction endonuclease are shown in Table 1. In the case of long size fragment, *Kpn*I enzyme showed the highest followed by *Nde*I and *Xba*I. However, none of the enzymes were found to produce more than 100 kb fragment size. Based on the rest parameters *Kpn*I was found as the most feasible and effective restriction enzyme to have the maximum MapSets for OM process (Table 1).

Optical maps construction and assembly

Based on the efficiency of the enzyme, HMW DNA was digested with *Kpn*I (New England Biolab, USA) restriction enzyme on the optical chips followed by subsequent dyeing that generated 71 GB raw data from 19 MapCards (Table 2). A total of 270,343 Single-Molecule Restriction Fragments (SMRMs) with an average size of 263.22 kb were produced from the optical chips analysis (Table 2). Within total molecules, 5,007,936 fragments were found having an average size of 14.209 kb. In addition to this, assembly of the molecules produced 12 unambiguous super-scaffolds (denoted as chromosomes) that are terminated with telomeric blunt ends (Figure 2). The

Table 1. Evaluation of restriction enzyme for OM compatibility of *Macrophomina phaseolina* MS 6 genome.

Enzyme	5-20 kb (%)	6-15 kb (%)	6-12 kb (%)	AFS (kb)	Frags>100 kb	Max. frag. size (kb)
*Af*1II	92.57	77.95	52.92	4.94	0	87.74
*Bam*H	21.92	3.87	3.87	4.77	0	39.95
***Kpn*I**	**98.69**	**79.79**	**74.42**	**7.80**	**0**	**63.25**
*Nco*I	0.00	0.00	0.00	3.23	0	31.09
*Nhe*I	87.55	55.88	55.88	5.29	0	64.05
*Spe*I	61.46	32.89	16.44	6.00	3	120.01
*Bg*1II	14.26	5.08	5.08	3.70	0	46.13
*EcoR*I	36.76	10.36	10.36	4.54	0	36.13
*Mlu*I	58.76	21.97	21.97	4.83	0	52.64
*Nde*I	95.10	70.03	68.78	7.55	0	55.90
*Pvu*II	0.00	0.00	0.00	2.41	0	31.09
*Xba*I	94.60	86.07	71.24	3.48	0	67.38
*Xho*I	0.00	0.00	0.00	2.70	0	25.67

Table 2. Optical map construction statistics.

Parameter	Maps
Number of molecules	270,343
Average molecule size (kb)	263.216
Number of fragments	5,007,936
Average fragment size (kb)	14.209
Total size (Mb)	71,158,564
Average quality score	0.517

chromosomes ranged from 1.6 to 6.7 Mb in sizes and spanned a total 49.723 Mb by joining all SMRMs through Argus^{TM} optical mapping system. It was also observed that optical mapping reduced the number of scaffolds from 94 to 88 where the largest scaffold increased by ~2 Mb in size. The indications in terms of contiguity along with quality and improvement, N50 placed on scaffold number 5 of OM instead of scaffold 6 of NGS. It increased by 4.25 Mb from 3.39 of NGS. Correspondingly, N90 also changed over its place on scaffold 11 instead of 14 by increasing size 2.9 Mb from 1.4. Although the N rate increased by 0.09%, still the GC content was unchanged (Table 3).

These results clearly pointed out the improvement of the assembly quality of *M. phaseolina* MS6 genome.

Alignment features between optical maps and reference maps

In alignment matrix, among all scaffolds only 17 were anchored on 11 chromosomes that spanned over 93.31% of the genome while none were on chromosome 12 (Figures 3 and 4). The sizes of aligned and non-aligned scaffolds were 46.35 and 3.3 Mb, respectively. Optical mapping deciphered 107 gaps and 4 overlaps size totaling 9.98 Mb and 26,040 kb, respectively, while 18% of the gaps can be closeable (Table 4).

A total number of 12 inversions (map is in reverse orientation) having 17.07 Mb in size were identified and made corrected for sequence finishing (Supplementary Files 1 and 2).

DISCUSSION

We have constructed whole-genome optical maps of M. phaseolina MS6 genome based on HMW DNA shearing by KpnI restriction endonuclease using OpGenTM optical mapping approach.. This technology is used to pick out the different types of incongruity between sequence generated *in silico* map and optical map along with current sequence validation of total spanned assembly. These issues were achieved by a series of action like-(i) alignment of optical map with *in silico* restriction map to find out all types of error, (ii) characterization of sequence contigs in respect to finding out the gaps, and (iii) the sequences were validated and placed on the optical map resulting in an explicit sequence validation. In the optical mapping process, restriction endonuclease is used to

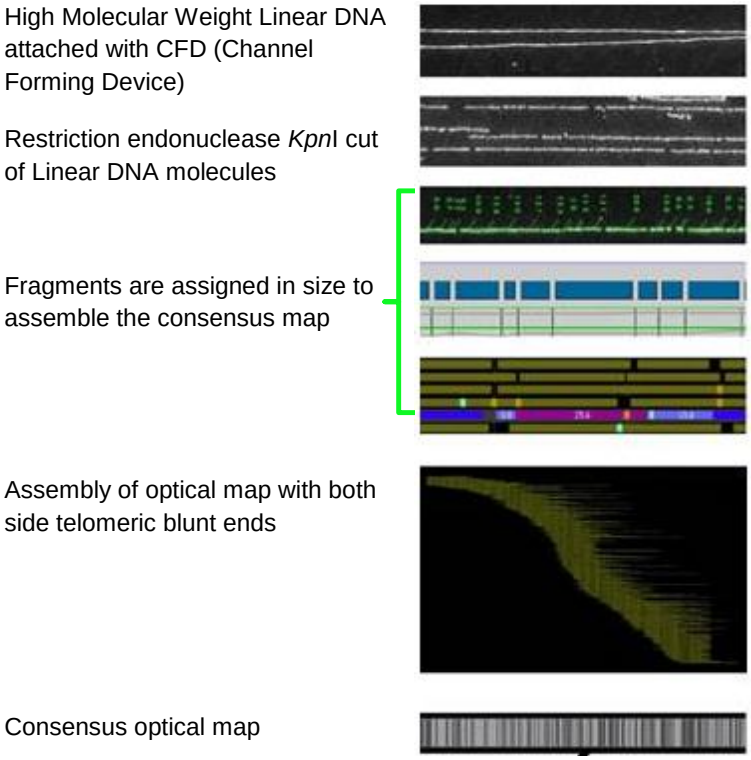

Figure 1. Generation of optical map from High Molecular Weight (HMW) DNA of *Macrophomina phaseolina* MS6.

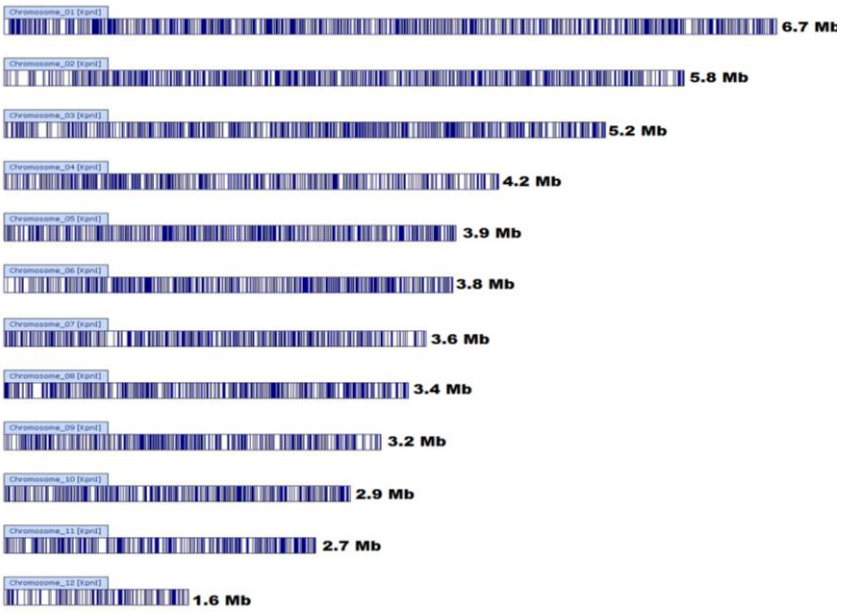

Figure 2. Optical maps of 12 chromosomes. Each vertical line represents the *Kpn*I restriction site. Chromosomes are ranked based on size.

digest total genomic DNA as a single molecule of >200 kb in size that was assembled into an ordered high-resolution restriction map possessing all fundamental genomic bases (Reslewic et al., 2005). This iterative and

Table 3. Assembly improvement statistics.

Parameter	Before (NGS)	After (OM)
No. of scaffolds	94	88
Largest scaffold (bp)	5,651,736	7,569,616
Bases in scaffolds (bp)	49,679,705	49,723,705
N50 scaffold	6	5
N50 length (bp)	3,400,455	4,259,500
N90 scaffold	14	11
N90 length (bp)	1,465,625	2,920,785
GC content	52.43%	52.43%
N rate	2.33%	2.42%

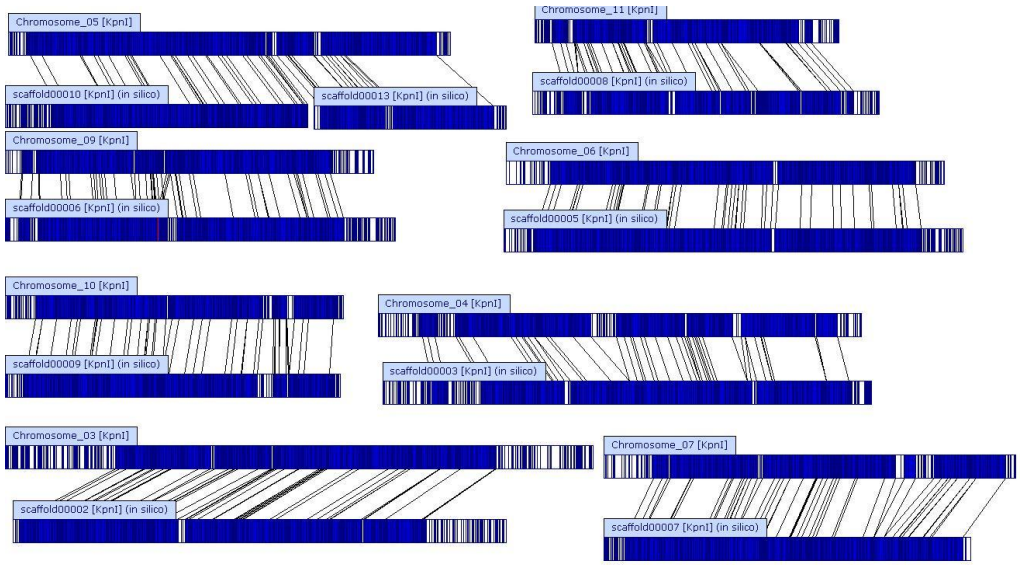

Figure 3. Alignment between optical maps and NGS sequence generated scaffolds. The blue-shaded regions of each map represent regions of the genomes that are similar whereas white areas are different. The alignment lines (lines connecting maps) connect regions of similarity from one map to the other.

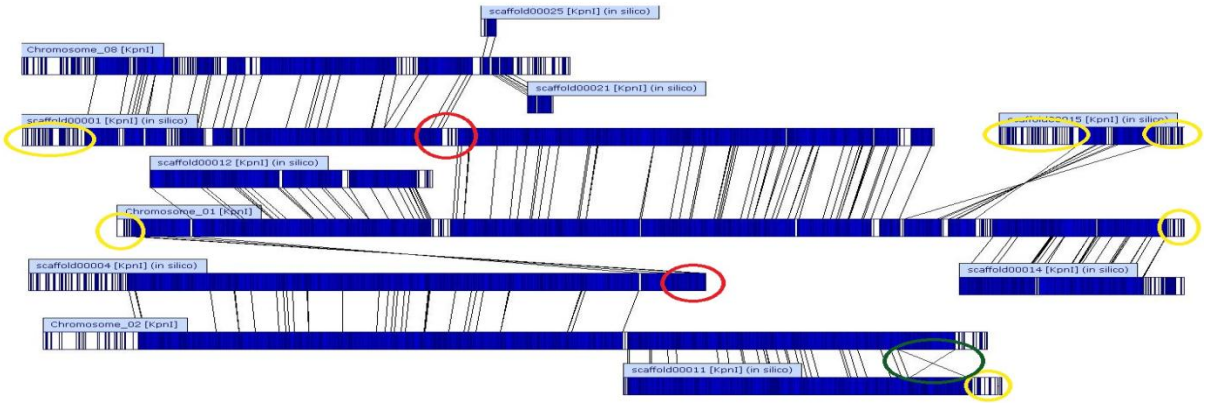

Figure 4. Alignment derived discordances of *de novo* chromosomes and NGS scaffolds. Regions encircled in red, crossed green and yellow indicates misassembled, inversion and low-quality assembly, respectively.

Table 4. Sequence alignment statistics.

Parameter	Alignment
Number of aligned scaffold	17
The total size of aligned scaffolds	46356277
Genome covered (%)	93.31
Number of unaligned scaffold	77
Unaligned sequences (%)	6.69
Total size of unaligned contigs	3323428
Number of total gaps	107
Number of gaps over 2 kb	91
Closeable gaps (%)	18
The total number of contig overlaps	4

computational assembly process joined all the SMRMs into super scaffolds by accomplishing sufficient representation across the chromosomes by coverage, the sufficient number of molecule maps covering each restriction fragment of the chromosome (depth >30X) and represents the genome complexity in terms of composition and structure (Ghurye and Pop, 2019). Finally, the optical mapping process generated 12 chromosomes terminating with both chromosomal telomeric blunt ends (blunt ends are not enzyme cut sites rather than the true end of a chromosome where the SMRMs ended at the same sequence). Furthermore, the telomeric end sequence (TTAGGG) of filamentous fungi within optical mapping organized ordered chromosomal sequence and found every chromosome possess their telomeric repetitive nucleotide sequence in between last SMRMs's (Average length 263 kb). The whole-genome restriction map consists of the chromosomes with dispersed arranged gaps. The similar results were observed in rice (Zhou et al., 2007) where the physical map consists of 14 contigs, covering its 12 chromosomes. In *Ganoderma lucidum* (Chen et al., 2012), 82 scaffolds were ordered and oriented onto 13 chromosome-wide optical maps that are very similar to our optical mapping results. The finished current NGS assembly is 49.295 Mb, that is, very close to our estimated 49.723 Mb *Kpn*I optical restriction map. The difference between the two assemblies was around ~1% which is identified as map error denoted as gaps, misassembles, and inversions. The improved and furnished assembly along with chromosome evolution mannered study of the fungus might be used for valuable resources along with fixation of control measures by biotechnological manner.

Conclusion

Here we presented an improved assembly of *M. phaseolina* MS6 genome with chromosomal level analyses using optical mapping data was presented. *In silico* analyzed 12 chromosomes with congruence and discordance makes the assembly error-free. The improved non-erroneous longer scaffold based chromosomal spanned assembly might be considered as the milestone to researchers for searching precisely its pestilential tools as well as survival dimensions in diverse environmental cues. The furnished assembly can also be used for future chromosomal re-arrangements and evolution studies in other fungi along with its control measures by developing the cross-talk phenomena between host and the pathogen.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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SUPPLEMENTARY MATERIALS

File 1. Optical mapping gaps and overlaps

Optical Map	Type	Start	End	Length
Chromosome_01 [KpnI]	Gap	1	79358	79358
Chromosome_01 [KpnI]	Overlap	447422	460046	12625
Chromosome_01 [KpnI]	Gap	747528	752122	4595
Chromosome_01 [KpnI]	Gap	945314	950401	5088
Chromosome_01 [KpnI]	Gap	1290151	1292842	2692
Chromosome_01 [KpnI]	Gap	1923102	2049281	126180
Chromosome_01 [KpnI]	Gap	2538197	2541665	3469
Chromosome_01 [KpnI]	Gap	3196744	3213241	16498
Chromosome_01 [KpnI]	Gap	3657504	3660489	2986
Chromosome_01 [KpnI]	Gap	4010741	4012829	2089
Chromosome_01 [KpnI]	Gap	4150231	4179968	29738
Chromosome_01 [KpnI]	Gap	4634834	4677569	42736
Chromosome_01 [KpnI]	Gap	4795755	4818973	23219
Chromosome_01 [KpnI]	Gap	5050756	5094873	44118
Chromosome_01 [KpnI]	Gap	5258586	5362643	104058
Chromosome_01 [KpnI]	Gap	5726673	5732010	5338
Chromosome_01 [KpnI]	Gap	5996370	6005240	8871
Chromosome_01 [KpnI]	Gap	6434333	6581957	147625
Chromosome_02 [KpnI]	Gap	1	576503	576503
Chromosome_02 [KpnI]	Gap	3566337	3574343	8007
Chromosome_02 [KpnI]	Gap	4666099	4667788	1690
Chromosome_02 [KpnI]	Gap	4706528	4708564	2037
Chromosome_02 [KpnI]	Overlap	5236138	5240657	4520
Chromosome_02 [KpnI]	Gap	5598673	5790302	191630
Chromosome_03 [KpnI]	Gap	1	831165	831165
Chromosome_03 [KpnI]	Gap	1327249	1329064	1816
Chromosome_03 [KpnI]	Gap	1694143	1696119	1977
Chromosome_03 [KpnI]	Gap	2788280	2802124	13845
Chromosome_03 [KpnI]	Gap	3296156	3337159	41004
Chromosome_03 [KpnI]	Gap	3741990	3743910	1921
Chromosome_03 [KpnI]	Gap	3901113	3913498	12386
Chromosome_03 [KpnI]	Gap	4176741	5119287	942547
Chromosome_04 [KpnI]	Gap	1	387731	387731
Chromosome_04 [KpnI]	Gap	499599	678302	178704

File 1. Contd.

Chromosome_04	[KpnI]	Gap	1160359	1194969	34611
Chromosome_04	[KpnI]	Gap	1249413	1251114	1702
Chromosome_04	[KpnI]	Gap	1853551	2077590	224040
Chromosome_04	[KpnI]	Gap	2285405	2287395	1991
Chromosome_04	[KpnI]	Gap	2669156	2688624	19469
Chromosome_04	[KpnI]	Gap	3080799	3166130	85332
Chromosome_04	[KpnI]	Gap	3331794	3338912	7119
Chromosome_04	[KpnI]	Gap	3807654	3817821	10168
Chromosome_04	[KpnI]	Gap	4005328	4212496	207169
Chromosome_05	[KpnI]	Gap	1	113512	113512
Chromosome_05	[KpnI]	Gap	1126000	1189809	63810
Chromosome_05	[KpnI]	Gap	1528950	1562392	33443
Chromosome_05	[KpnI]	Gap	1601451	1610620	9170
Chromosome_05	[KpnI]	Gap	1769933	1772111	2179
Chromosome_05	[KpnI]	Gap	3211560	3228069	16510
Chromosome_05	[KpnI]	Gap	3664142	3849511	185370
Chromosome_06	[KpnI]	Gap	1	384283	384283
Chromosome_06	[KpnI]	Gap	639443	641244	1802
Chromosome_06	[KpnI]	Gap	2001087	2031642	30556
Chromosome_06	[KpnI]	Gap	2323247	2374952	51706
Chromosome_06	[KpnI]	Gap	3453531	3454828	1298
Chromosome_06	[KpnI]	Gap	3579468	3820145	240678
Chromosome_07	[KpnI]	Gap	1	72040	72040
Chromosome_07	[KpnI]	Gap	714355	874226	159872
Chromosome_07	[KpnI]	Gap	967769	1051336	83568
Chromosome_07	[KpnI]	Gap	1852132	1854424	2293
Chromosome_07	[KpnI]	Gap	2207992	2254218	46227
Chromosome_07	[KpnI]	Gap	3011557	3036031	24475
Chromosome_07	[KpnI]	Gap	3157829	3590256	432428
Chromosome_08	[KpnI]	Gap	1	543109	543109
Chromosome_08	[KpnI]	Gap	737294	793992	56699
Chromosome_08	[KpnI]	Overlap	904383	906268	1886
Chromosome_08	[KpnI]	Gap	1021335	1152669	131335
Chromosome_08	[KpnI]	Gap	1252737	1304792	52056
Chromosome_08	[KpnI]	Gap	1463069	1558040	94972
Chromosome_08	[KpnI]	Gap	2380415	2514290	133876

File 1. Contd.

Chromosome_08	[KpnI]	Gap	2847035	2906972	59938
Chromosome_08	[KpnI]	Gap	2964044	2971980	7937
Chromosome_08	[KpnI]	Overlap	3012351	3019359	7009
Chromosome_08	[KpnI]	Gap	3111589	3444310	332722
Chromosome_09	[KpnI]	Gap	1	125892	125892
Chromosome_09	[KpnI]	Gap	235466	283656	48191
Chromosome_09	[KpnI]	Gap	524756	526744	1989
Chromosome_09	[KpnI]	Gap	550524	552683	2160
Chromosome_09	[KpnI]	Gap	1097161	1127720	30560
Chromosome_09	[KpnI]	Gap	1219548	1220850	1303
Chromosome_09	[KpnI]	Gap	1271646	1287701	16056
Chromosome_09	[KpnI]	Gap	1361684	1408860	47177
Chromosome_09	[KpnI]	Gap	2094379	2096211	1833
Chromosome_09	[KpnI]	Gap	2515039	2531652	16614
Chromosome_09	[KpnI]	Gap	2703610	2705653	2044
Chromosome_09	[KpnI]	Gap	2724149	2725923	1775
Chromosome_09	[KpnI]	Gap	2839562	3209463	369902
Chromosome_10	[KpnI]	Gap	1	262536	262536
Chromosome_10	[KpnI]	Gap	632659	652206	19548
Chromosome_10	[KpnI]	Gap	795495	803422	7928
Chromosome_10	[KpnI]	Gap	1183685	1185635	1951
Chromosome_10	[KpnI]	Gap	1398164	1453469	55306
Chromosome_10	[KpnI]	Gap	1711265	1713480	2216
Chromosome_10	[KpnI]	Gap	2242393	2335381	92989
Chromosome_10	[KpnI]	Gap	2385276	2386923	1648
Chromosome_10	[KpnI]	Gap	2451049	2518758	67710
Chromosome_10	[KpnI]	Gap	2862713	2948255	85543
Chromosome_11	[KpnI]	Gap	1	234895	234895
Chromosome_11	[KpnI]	Gap	315293	352704	37412
Chromosome_11	[KpnI]	Gap	461982	469634	7653
Chromosome_11	[KpnI]	Gap	1260397	1282191	21795
Chromosome_11	[KpnI]	Gap	1618703	1682423	63721
Chromosome_11	[KpnI]	Gap	1704229	1748358	44130
Chromosome_11	[KpnI]	Gap	1872927	1874668	1742
Chromosome_11	[KpnI]	Gap	1897448	1899532	2085
Chromosome_11	[KpnI]	Gap	2062058	2067777	5720
Chromosome_11	[KpnI]	Gap	2186940	2200017	13078
Chromosome_11	[KpnI]	Gap	2223138	2224929	1792
Chromosome_11	[KpnI]	Gap	2307985	2377282	69298
Chromosome_11	[KpnI]	Gap	2503819	2652226	148408

File 2. Alignment information between optical maps and NGS sequence derived insilico maps

Chromosome	Start	End	Contig	Start	End	Orientation
Chromosome_01 [KpnI]	84599	447421	scaffold00004 [KpnI] (in silico)	3754787	4138122	-1
Chromosome_01 [KpnI]	460047	1919768	scaffold00012 [KpnI] (in silico)	6677	1630180	1
Chromosome_01 [KpnI]	2058751	4621849	scaffold00001 [KpnI] (in silico)	2739187	5428339	1
Chromosome_01 [KpnI]	4681571	4793752	scaffold00001 [KpnI] (in silico)	5520902	5639258	1
Chromosome_01 [KpnI]	4821388	5046981	scaffold00015 [KpnI] (in silico)	708429	946091	-1
Chromosome_01 [KpnI]	5096608	5252281	scaffold00015 [KpnI] (in silico)	488655	651614	-1
Chromosome_01 [KpnI]	5369160	6431276	scaffold00014 [KpnI] (in silico)	6517	1129657	1
Chromosome_02 [KpnI]	584978	3553334	scaffold00004 [KpnI] (in silico)	614339	3738177	1
Chromosome_02 [KpnI]	3583285	5236137	scaffold00011 [KpnI] (in silico)	23765	1746602	1
Chromosome_02 [KpnI]	5240658	5591437	scaffold00011 [KpnI] (in silico)	1754726	2110127	-1
Chromosome_03 [KpnI]	838070	4161621	scaffold00002 [KpnI] (in silico)	52587	3570054	-1
Chromosome_04 [KpnI]	389545	494988	scaffold00003 [KpnI] (in silico)	3722076	3833917	-1
Chromosome_04 [KpnI]	680721	4002755	scaffold00003 [KpnI] (in silico)	196387	3397557	-1
Chromosome_05 [KpnI]	121869	1514309	scaffold00013 [KpnI] (in silico)	51660	1560437	-1
Chromosome_05 [KpnI]	1563606	3660695	scaffold00010 [KpnI] (in silico)	443499	2633246	-1
Chromosome_06 [KpnI]	386252	3571691	scaffold00005 [KpnI] (in silico)	284719	3635033	1
Chromosome_07 [KpnI]	88203	3155720	scaffold00007 [KpnI] (in silico)	187125	3128052	-1
Chromosome_08 [KpnI]	544757	733814	scaffold00001 [KpnI] (in silico)	451658	650302	1
Chromosome_08 [KpnI]	796028	904382	scaffold00001 [KpnI] (in silico)	694057	808998	1
Chromosome_08 [KpnI]	906269	1016062	scaffold00001 [KpnI] (in silico)	825646	937173	1
Chromosome_08 [KpnI]	1157124	1250126	scaffold00001 [KpnI] (in silico)	1057655	1156833	1
Chromosome_08 [KpnI]	1344489	1455367	scaffold00001 [KpnI] (in silico)	1235932	1348893	1
Chromosome_08 [KpnI]	1560874	2844475	scaffold00001 [KpnI] (in silico)	1436060	2636783	1
Chromosome_08 [KpnI]	2909815	2955214	scaffold00025 [KpnI] (in silico)	37991	85335	1
Chromosome_08 [KpnI]	2971981	3012350	scaffold00021 [KpnI] (in silico)	1	42763	1
Chromosome_08 [KpnI]	3019360	3100473	scaffold00021 [KpnI] (in silico)	62711	144842	1
Chromosome_09 [KpnI]	139286	233651	scaffold00006 [KpnI] (in silico)	118062	217210	1
Chromosome_09 [KpnI]	287540	2833740	scaffold00006 [KpnI] (in silico)	296554	2946891	1
Chromosome_09 [KpnI]	1291111	1357055	scaffold00006 [KpnI] (in silico)	1321185	1392070	1
Chromosome_10 [KpnI]	264954	2857186	scaffold00009 [KpnI] (in silico)	174737	2825737	1
Chromosome_11 [KpnI]	245190	300565	scaffold00008 [KpnI] (in silico)	2739572	2797367	-1
Chromosome_11 [KpnI]	357185	2499966	scaffold00008 [KpnI] (in silico)	280656	2680529	-1

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Full Length Research Paper

Callus induction in three mosaic disease resistant cassava cultivars in Benin and genetic stability of the induced calli using simple sequence repeat (SSR) and sequence-characterized amplified region (SCAR) markers

Amitchihoué Franck Sessou[1], Jane W. Kahia[2*], Elijah Ateka[3], Jerome Anani Houngue[4], Colombe Dadjo[1], Peter Njenga[3] and Corneille Ahanhanzo[4]

[1]Institute of Basic Sciences, Technology and Innovation, Pan African University, P. O. Box 62000-00200, Nairobi, Kenya.
[2]Coffee Research Institute, P. O. Box 4, Ruiru, Kenya.
[3]Jomo Kenyatta University of Agriculture and Technology, P. O. Box 62000-00200, Nairobi, Kenya.
[4]Laboratory of Biochemistry and Molecular Biology, Faculty of Sciences and Technology (FAST), University of Abomey-Calavi (UAC), P.O. Box 526 Cotonou, Benin.

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The effect of different concentrations of thidiazuron (TDZ), benzyl amino purine (BAP), kinetin and 2,4-dichlorophenoxy acetic acid (2, 4-D) on callus induction in three elite cassava cultivars (agric-rouge, atinwewe and agblehoundo) was evaluated. Leaf explants harvested from greenhouse-grown cassava were sterilised using different concentrations of commercial bleach commonly called Jik (3.85% NaOCl) at different time intervals. The highest number (94%) of clean explants was obtained when 2% (v/v) Jik was used for 15 min. The explants were cultured in half MS media supplemented with different growth regulators TDZ, BAP, kinetin 2, 4-D, 100 mg/l myo inositol, 2% sucrose and gelled with 0.3% phytagel. Callus formation was observed from the cut edges of the leaves in all cultivars after 10 days in medium supplemented with TDZ, 12 days in BAP medium, and 15 days in kinetin medium. There were significant ($p < 0.05$) differences in callus formation among all cytokinins types and concentrations. However, there were no significant differences in callus formation in different 2,4-D concentrations. All 2,4-D concentrations produced 100% callus in all the cultivars. However, 2,4-D at 2 µM significantly produced the highest (2.48±0.30) callus weight in cultivar atinwewe. Furthermore, simple sequence repeats (SSR) and sequence-characterized amplified region of the induced calli on TDZ and 2,4-D media indicated the loss of CMD2 gene among induced calli compared to the mother plants.

Key words: Cassava, simple sequence repeats (SSR) and sequence-characterized amplified region (SCAR), genetic stability, callus, cytokinins, auxins.

INTRODUCTION

Cassava (*Manihot esculenta Crantz*) is the most important root crop in the tropics and ranks fourth after rice, wheat and corn as a source of calories for human consumption (Acedo and Labana, 2008). In Benin, cassava is consumed by more than 54% of the population in different ways as raw or after processing into gari, chips or tapioca (MAEP, 2013). This is an indication of its importance as food security crop and

poverty alleviation in the country. The cultivation of cassava in Benin is facing many challenges which include pests and diseases, lack of good agronomic practices, land degradation, shortage of planting materials, limited processing options etc. (Agre et al., 2015). Viral diseases, particularly cassava mosaic disease (CMD) are the most economically important and may lead to yield losses of 20 to 95% (Fauquet et al., 1990; Hahn et al., 1989). Due to these constraints, the average crop yield in Benin is low (15.55 tonnes per hectare) compared to the global yield of 90 tonnes (FAOSTAT, 2017). Recent breeding work in Benin has resulted in the release of some local cultivars (Agric-rouge, Atinwewe and Agblehoundo) that are resistant to cassava mosaic disease (CMD) (Houngue et al., 2018). The cultivars contain CMD2 gene which is dominant monogenic resistance locus (Fondong, 2017). The propagation of those cassava cultivars in the field by farmers is by cuttings and few works have been done for in vitro culture (Cacaï et al. 2012, 2013). The traditional method by cuttings is not only limiting in the numbers of planting materials but is also cumbersome, and labour intensive. Therefore, there is need to evaluate alternative propagation methods that are fast and tissue culture offers a feasible option.

In tissue culture technique, somatic embryogenesis has an advantage over micropropagation in that it generates a new plant with both root and shoot meristems from actively dividing somatic cells in the same step and within a short time period, whereas micropropagation requires additional steps and a longer time frame (Leva et al., 2012). Although regeneration of microshoots from cassava nodes can be achieved with a high rate of success, somatic embryogenesis is still preferred to microshoot propagation technique for two main reasons: High multiplication rates, and the possibility of initiating cultures from readily available and renewable leaf explants. One of the advantages of using somatic embryogenesis as a method for mass propagation is that the embryos have concomitant development of both root and shoot meristems which, under optimal conditions, can grow synchronously to produce normal plants. This procedure can save a great deal of time in those situations where conventional vegetative propagation is slow or difficult to carry out and manage.

The induction of somatic cells from leaf explants during tissue culture requires the explants sterilization using different sterilants. The type of sterilizing agent used depends on the source and the type of the explants and the purpose of the experiments. There are few studies on sterilization of cassava leaf explants from greenhouse. For instance, Danso (1997) reported the use of alcohol to sterilize young leaf of four weeks old shoot tips from greenhouse cassava. On the other hand, Magaia (2015) reported 100% clean explants when cassava leaf from greenhouse were sterilized with 70% ethanol for 2 min followed by 0.05% of mercuric chloride for 1 or 2 min. During the current study, the effect of Jik (3.85% NaOCl) on sterilization of leaf explants was evaluated. Somatic embryogenesis have been reported to be suitable for mass propagation of highly performing cassava clones (Osorio et al., 2012). Many studies have been done in callus induction in several crops using cytokinins and auxins. For example, Faye et al. (2015) and Mongomake et al. (2015) reported callus formation with MS media supplemented with cytokinins in some cassava cultivars. Phua et al. (2016) and Fathil et al. (2017) reported that 2,4-D at low (0.25 - 1 mg/L) concentrations produced 100% callus in *Clinacanthus nutans* and *Citrus suhuiensis.* Furthermore, Castro et al. (2016) reported in *Byrsonima verbascifolia* an excellent (100%) callus induction using 2,4-D combined with BAP. Callus-derived somaclonal variation based on morphological and biochemical parameters have been reported by Pajević et al. (2004) in sunflower, Jibu et al. (2006) in tea, Rajeswari et al. (2009) in Sugarcane, Park et al. (2010) in Rice, Beyene et al. (2016) and Chauhan et al. (2018) in cassava. Although, many works have generated cassava plantlets from somatic embryogenesis in different cassava varieties (Anuradha et al., 2015; Atehnkeng et al., 2006; Feitosa et al., 2007; Le et al., 2007; Vidal et al., 2014), there are no reports on such work on cassava varieties in Benin. Even in cases where regeneration of callus has been reported there is hardly any information on evaluating the stability of the regenerated callus. Callus formation is an important requirement for establishing embryogenic culture, plantlet regeneration and germplasm conservation.

Microsatellite-based marker techniques such as Simple sequence repeat (SSR) and sequence-characterized amplified region (SCAR) markers have successfully been used in the detection of CMD2 resistant gene in cassava (Okogbenin et al., 2008; Houngue et al., 2018). SSR markers have also been used in detecting genetic differences or similarities in several micropropagated plants, including cotton (Jin et al., 2008) and medicinal plants such as *Jatropha curcas* (Sun et al., 2008). The high reproducibility, simplicity, and low cost of the experimental procedures of SSR and SCAR compared to other molecular markers such as restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) makes them more appropriate for such studies. The aim of the study was to determine the optimal sterilization technique for cassava leaf explants. The effects of cytokinins (TDZ, kinetin and BAP) and 2,4-D concentrations on frequency of callus

*Corresponding author. E-mail: janekahia@yahoo.co.uk. Tel: +254723774727.

Table 1. Specific SSR and SCAR primers used for detection of CMD2 resistant gene in cassava mother plants and induced calli.

Primer code	Marker system	Forward primer sequence	Reverse primer sequence	Expected sequence length (bp)	Annealing temperature (˚C)
NS169	SSR	GTGCGAAATGGAAATCAATG	GCCTTCTCAGCATATGGAGC	319	55
RME1	SCAR	AGAAGAGGGTAGGAGTTATGT	ATGTTAATGTAATGAAAGAGC	700	55

formation, weight of callus induced from greenhouse-grown leaf explants of three mosaic resistant cassava cultivars (Agric-rouge, Atinwewe and Agblehoundo) and finally to evaluate the 'CMD2 conformity' of the calli using SSR and SCAR markers.

MATERIALS AND METHODS

Cassava cuttings of three (Agric-rouge, Atinwewe and Agblehoundo) cultivars resistant to CMD were collected from the southern region in Republic of Benin and transported to Coffee Research Institute (CRI), Ruiru-Kenya where the tissue culture studies were carried out. The molecular analysis work was done in the Molecular Biology and Biotechnology laboratories of CRI and Pan African University of Basic Sciences, Technology and Innovation (PAUSTI), Kenya.

Explants sterilization

Leaf explants from three weeks old stem cuttings were harvested and transported from the greenhouse to the laboratory in a beaker containing tap water. Once in the laboratory, they were cleaned with cotton wool containing liquid soap to remove any surface debris and rinsed with tap water. They were then subjected to sterilization under the lamina flow hood using 2 and 5% v/v commercial bleach (Jik) for 5, 10, 15 and 20 min. After exposure to the sterilant, the explants were rinsed two times with sterile distilled water and thereafter given a quick immersion (30 s) in 70% (v/v) ethanol and finally rinsed four times with sterile distilled water. The leaf explants were trimmed to approximately 1 cm^2 leaf discs and cultured individually in tubes containing hormone free MS media. The cultures were incubated in a dark room at a temperature of 25 ± 2˚C. Data were collected after 4, 8, and 15 days on the percent clean explants. This was calculated as total number of contaminated explants / total number of explants x 100.

Callus induction and culture conditions

The media used to evaluate the effect of cytokinins and auxin on callus induction from leaf explants was half-strength MS (Murashige and Skoog, 1962) supplemented with either BAP or kinetin (5, 10, 20 and 40 µM), thidiazuron (0.1, 0.5, 1 and 1.5 µM), or 2, 4-D (2, 5, 10, 25 and 30 µM), 100 mg/L inositol, 2% (w/v) sucrose and gelled with 0.3% Phytagel in separate experiments. The media pH was adjusted to 5.7 by using either 1 N HCl or 1 N NaOH before the gelling agent was added. The media was dispensed in culture test tubes and autoclaved at a temperature of 121°C and a pressure of 1.1 $kg{\cdot}cm^2$ for 20 min. The cultures were incubated in a dark room maintained at 25 ± 2°C. Half-strength MS medium without growth regulators was used as control.

Subculture of callus and data collection

The calluses were transferred to MS medium without growth regulators (control) and MS media supplemented with 1, 3 and 5 µM GA_3 (embryos induction medium) to study their embryogenic competence. Data were collected on weekly basis on the explants with callus (expressed as % callus induced) and weight of the callus. The percent (%) callus induced was calculated as total number of explants with callus / total number of explants cultured x 100.

Experimental design and data analysis

All experiments were laid out in completely randomized design (CRD) with 10 replicates per treatment and the experiment repeated three times. The data (percentage and weight of the callus) were subjected to one-way analysis of variance and the significant differences between treatments means were assessed using MINITAB version 19 software. Tukey analysis at 5% level was performed to assess difference between means. Data were also subjected to analysis as graph by GraphPad Prism 7 Software.

Assessment of the presence of CMD2 gene in calli

PCR-based SSR and SCAR markers as described by Houngue et al. (2018) were used to assess the CMD2 conformity of the induced calli. The mother plants growing in the greenhouses did not show any symptoms of CMD and it was used as the control. The characteristics of the primers used are shown in Table 1.

DNA extraction and quantification

DNA was extracted from young leaves picked from cassava mother plants and the callus according to the method described by Diniz et al. (2005). DNA quality and quantity were determined with Genova Spectrophotometer (Model 7415 Nano, Vacutec, South Africa) and quality was also assessed on 1% (w/v) agarose gel. The extracted DNA samples were stored at -20°C for SSR and SCAR analysis.

Polymerase chain reaction (PCR) for scoring CMD2 resistant gene

The SSR and SCAR analysis were performed as described by Omingo et al. (2017). DNA samples were diluted to 10 ng/µl for SSR and SCAR analysis. A total of 100 ng of each DNA sample was used in PCR reactions. A reaction mix was prepared to include: 2.5 µl of buffer (10 x), 2.5 µl of $MgCl_2$ (25 mM), 3.5 µl of dNTPs (500 µM), 2 µl of SSR or SCAR (10 µM) reverse (1 µl) and forward (1 µl) primers, 0.2 µl of Taq polymerase 5 µl. The 25 µl PCR volume was incubated in a thermocycler (Model FFG02HSD, made in UK) set for the following amplification conditions: One cycle at 95°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1.5 min, extension at 72 °C for 10 min and was held at 4°C. The amplified products were electrophoresed in 2.3% agarose gel and then visualized in a UV trans-illuminator (Model M-26, Upland, CA 91786 U.S.A) after staining in ethidium bromide

Table 2. Effects of commercial bleach (Jik) on elimination of surface contamination from cassava leaf explants.

Jik concentration (%) v/v	Exposure time (min)	Percentage of clean explants (%)		
		4 days	9 days	15 days
2	5	97	93	88
2	10	93	77	77
2	15	100	100	94
2	20	92	89	89
5	2	90	80	80
5	5	97	90	90
5	10	100	97	88
5	15	100	89	89

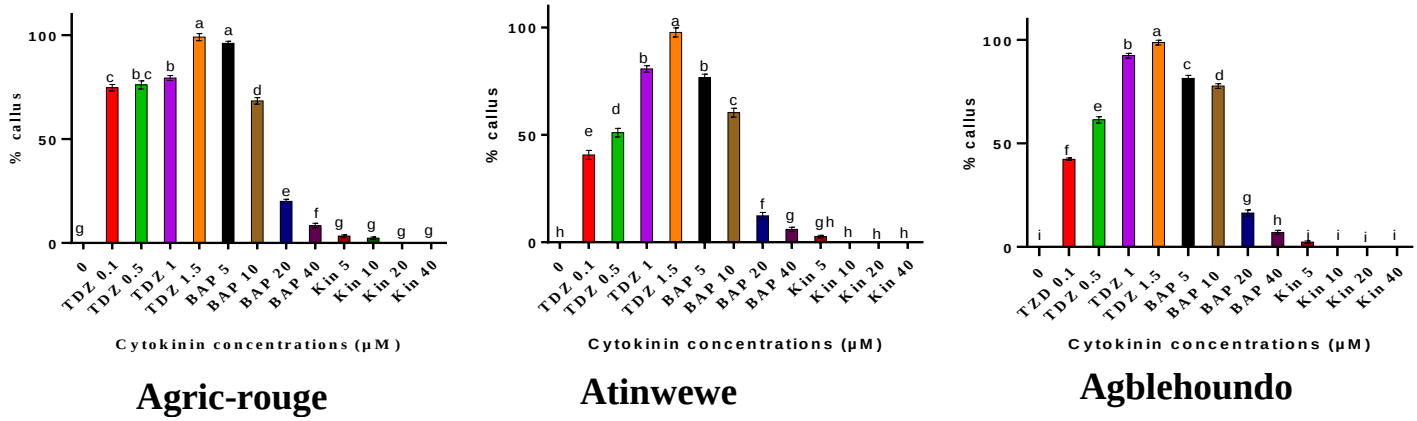

Figure 1. Effect of different cytokinin concentrations (µM) on callus formation from leaf explants of the three cultivars after one month of culture. *Means followed by the same letter are not significantly different at P≤0.05.

solution.

Scoring and analysis of bands

Amplified DNA fragments were run on agarose gel to score for the presence (1) or absence (0) of bands (Resistance gene) in the formed calli compared with mother plants. All reactions were repeated at least twice, and only distinct, reproducible, polymorphic and well-resolved bands across all runs were considered for analysis.

RESULTS

The result of the effect of Jik (3.85% NaOCl) on surface sterilization of cassava leaf explants are shown in Table 2. Most of the contaminations observed during this study were fungal (80%) while bacterial and yeast contaminants accounted for 20%. After 15 days the highest (97%) number of clean explants was obtained when the explants were sterilized in 2% Jik for 15 min and this sterilization procedure was used in all the subsequent experiments.

Effects of cytokinins on the callus formation

The results of the effects of different cytokinins on callus induction are shown in Figures 1 to 3. Callus formation was observed from the cut edges of the leaves in all the three cultivars. The cytokinin concentrations significantly ($p < 0.001$) affected induction of callus from the explants in all cultivars. Callus induction was first observed in all cultivars after ten days in medium supplemented with TDZ, 12 days in BAP medium, and 15 days in kinetin medium. It was generally observed that the media supplemented with TDZ 1.5 µM produced the highest percentage (98-99 %) explants with callus in all the three cultivars (Figure 1). It was also observed in all cultivars that increasing TDZ concentrations from 0.1 to 1.5 µM increased the percentage of callus production while increasing BAP concentrations from 5 to 40 µM decreased the production of callus. However, no significant difference ($p < 0.001$) was observed among kinetin concentrations and the control. Callusing of leaf explants began with curling of the tissue followed by swelling at the cut edges. The white calli turned to brown

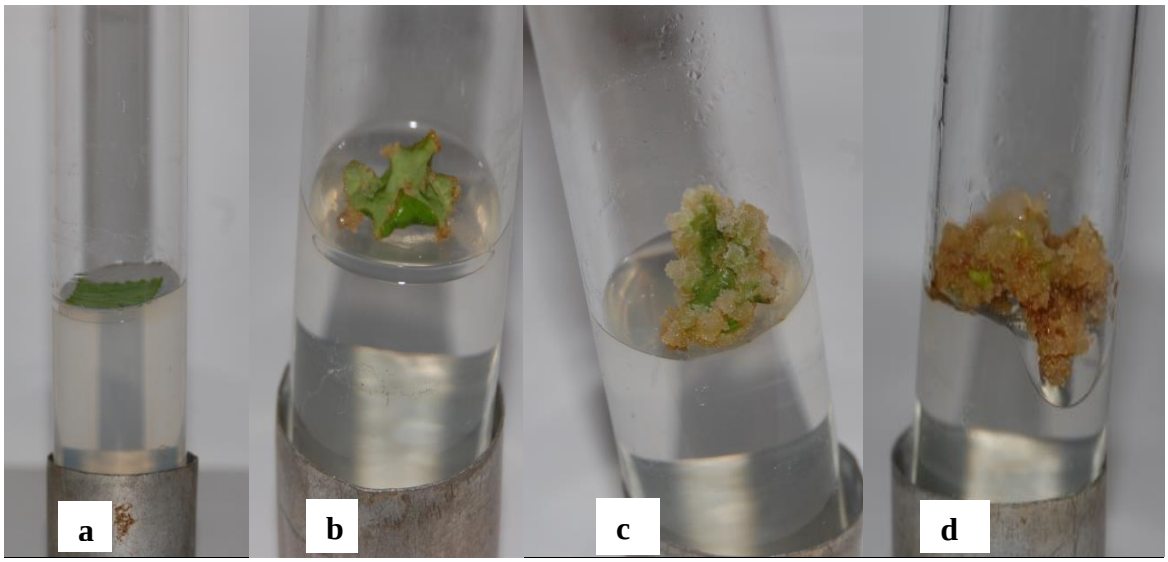

Figure 2. Effect of cytokinins on callus formation: a -Fresh culture, b - Initiation of callus 10 days after; c - White callus after 3 weeks; d - Callus turned light brown after one month.

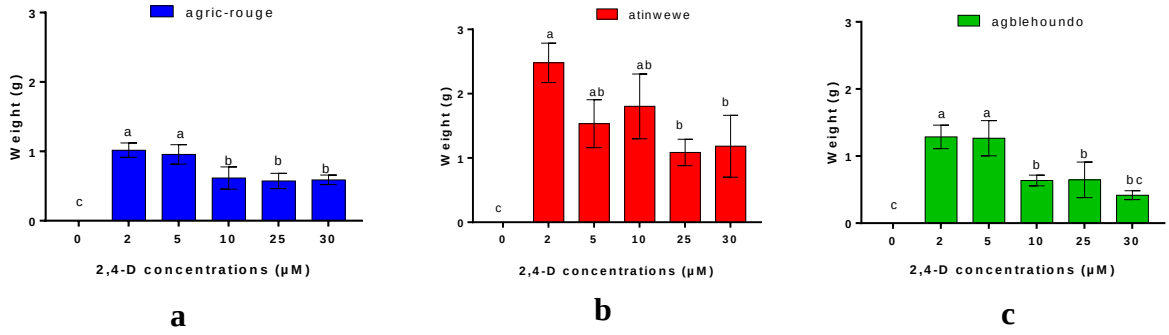

Figure 3. Effect of different 2, 4-D concentrations (µM) on callus formation from leaf explants: **a** - Agric-rouge, **b** - Atinwewe, and **c** - Agblehoundo cultivars after one month of culture. *Means followed by the same letter are not significantly different at P≤0.05.

after one month (Figure 2).

Effects of auxin 2,4-D on the callus formation

The results showed that there was no significant effect of the 2,4-D concentrations on callus induction in all the three cultivars. It was observed that all concentrations used in this study produced 100% frequency of callus in all cultivars after ten days. In agric-rouge and atinwewe cultivars, the first callus was observed after 4 days and after 6 days in agblehoundo. However, there was significance difference ($p < 0.001$) in the weight of the callus among all concentrations evaluated and across cultivars. Figure 3 shows the effect of 2,4-D on callus formation. Increasing concentration of 2, 4-D from 10 to 30 µM, reduced the weight of the callus in all cultivars. 2, 4-D 2 µM significantly produced the highest callus masses which 2.48 ± 0.30 atinwewe cultivar. There was no callus produced in the medium without growth regulators. The formed calli were white and friable in all cultivars. When the calli were transferred into media without growth regulators, 80% calli developed roots (Figure 4). An attempt to transfer the callus on a media supplemented with different concentration of GA_3 did not yield any embryos.

Assessment of the presence of CMD2 gene in calli

To confirm the presence of CMD2 gene in induced calli, PCR analyses were conducted. DNA fragments corresponding to the CMD2 gene (319 bp with SSR or 700 bp with SCAR) were amplified in all mother plants

Figure 4. Effect of auxin 2, 4-D on callus production: a - White formed callus after two weeks; b - Formation of root two months after callus was transferred to media without growth regulators.

whereas the corresponding band was not detected in some induced calli, indicating the loss of the resistance gene in the genome of those induced calli (Figures 5 and 6). In Figure 5, SSR primer revealed the loss of the resistant gene (319 bp) in calli induced from 2, 4-D supplemented media in cultivars Agric-rouge and Agblehoundo and in callus induced from TDZ medium in Agblehoundo. In Figure 6, SCAR primer revealed the presence of the resistant fragment (700 bp) in induced calli from both 2,4-D and TDZ media in cultivar agblehoundo.

DISCUSSION

Development of a sterilization technique for cassava leaf explants

The current study was conducted with an aim of optimizing the sterilization of leaf explants and callus induction using different growth regulators in the mosaic disease resistant cassava cultivars. The disinfectant widely used for surface sterilization is sodium hypochlorite (Miché and Balandreau, 2001). During the current study, Jik (NaOCl 3.85%) was found to be effective in sterilizing cassava leaf explants from greenhouse. The recorded highest (94%) number of clean explants was obtained when 2% Jik was used for 15 min. Similar results of high numbers of clean explants (100%) with greenhouse grown cassava leaf explants was reported by Magaia (2015) who used 0.1% $HgCl_2$ solutions. However, mercuric chloride is known to be highly toxic and may not be recommended for routine sterilization. The results of the current study concur with those of Guma et al. (2015) who reported high percentage (83%) of clean explants when greenhouse-grown leaf explants of anchote (*Coccinia abyssinica*) were exposed to 5% Jik for 10 min. Moreover, Jik was also found to be effective in sterilizing coffee leaf explants from the greenhouse (Lubabali, 2015).

Effects of different concentrations of cytokinins and auxins for regenerating plantlets through somatic embryogenesis

Cytokinins are known to stimulate cells and, as such, they are also suitable candidates for induction of somatic embryogenesis and caulogenesis (Deo et al., 2010). For example, thidiazuron (TDZ) has been reported to stimulate *in vitro* somatic embryogenesis (Srangsam and Kanchanapoom, 2003; Mithila et al., 2003; Lin et al., 2004; Chen and Chang, 2006; Mahendran and Bai, 2016; Mose et al., 2017). Cytokinins have been reported to induce callus formation in cassava (Faye et al., 2015; Mongomake et al., 2015) and in *Vitex doniana* (Dadjo et al., 2015). It was observed during the current study that all cytokinins failed to induce direct embryos from greenhouse leaf explants in all cassava cultivars. Instead, they produced callus and TDZ at 1.5 µM induced the highest callus percentage (98 to 99%) in all cultivars. The results of the current study also concur to those of Faye et al. (2015) and Mongomake et al. (2015) who reported callus formation with MS media supplemented with cytokinins in some cassava cultivars and also with Dadjo et al. (2015) who reported callus formation from *Vitex doniana* leaf explants cultured on media supplemented with cytokinins. The present report is however, contrary to that of Mongomake et al., (2015) who reported that medium supplemented with TDZ did not induce callus in some cassava cultivars. This could be probably due to the different genetic makeup. The current observation

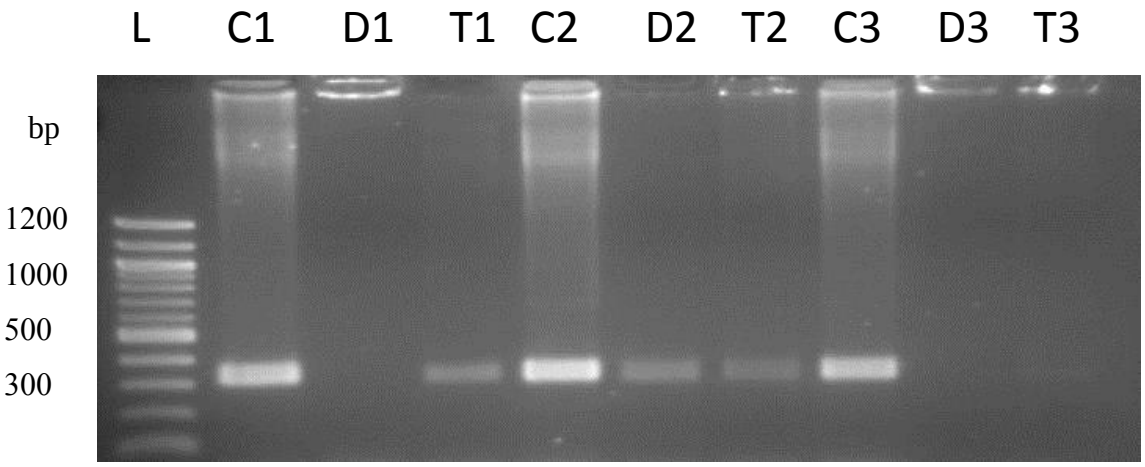

Figure 5. SSR banding pattern with primer NS169 in both *induced* calli (D1, D2, D3, T1, T2, and D3) and greenhouse-grown mother plants of agric-rouge (C1), atinwewe (C2), and agblehoundo (C3). D1, D2, and D3 were induced from leaf explants of C1, C2, and C3 cultured on 2,4- D media and T1, T2, and T3 were induced from leaf explants cultured of C1, C2, and C3 on TDZ media.

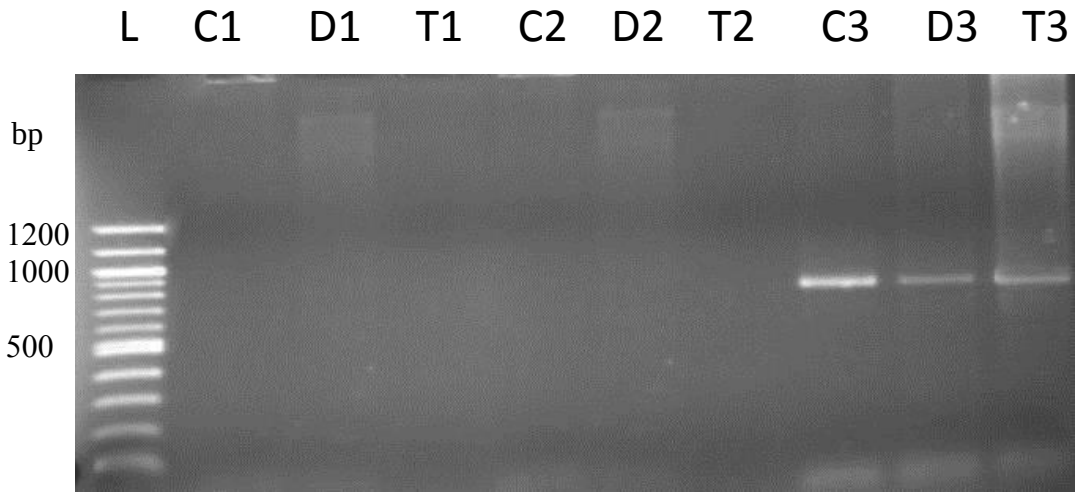

Figure 6. SCAR banding pattern with primer RME1 in both induced calli (D1, D2, D3, T1, T2, and D3) and greenhouse-grown mother plants of agric-rouge (C1), atinwewe (C2), and agblehoundo (C3). D1, D2, and D3 were induced from leaf explants of C1, C2, and C3 cultured on 2, 4-D media and T1, T2, and T3 were induced from leaf explants cultured of C1, C2, and C3 on TDZ media

concurs with Mongomake et al. (2015) and Faye et al. (2015) who reported that BAP produced better callus frequencies than kinetin in cassava.

The results of the current investigation revealed that auxin 2, 4-D at the range of 2 to 30 µM failed to induce somatic embryos from greenhouse- grown cassava leaf explants in all cultivars. These results are contrary to reports indicating that Murashige and Skoog medium supplemented with 2, 4-D was effective in induction of somatic embryos from young leaf lobes of *in vitro* grown shoots of cassava (Mathews et al., 1993; Ma and Xu, 2002; Le et al., 2007; Vidal et al., 2014; Mongomake et al., 2015). It was observed that 2, 4-D was highly efficient in inducing callus and all concentrations used in this study produced the 100% frequency of callus in the cultivars after ten days. Similar observations were made by Danso (1997), Fletcher et al. (2011) and Isah et al., (2018) who reported that 2, 4-D failed to induce direct embryos from greenhouse cassava leaf explants but it was best for callus formation. The results of the current study were similar to that of Phua et al. (2016) and Fathil et al. (2017), who reported that 2, 4-D at low (0.25 - 1 mg/L) concentrations produced 100% callus in *Clinacanthus nutans* and in *Citrus suhuiensis*. Similarly,

Castro et al. (2016) reported in *Byrsonima verbascifolia* a maximal callus induction (100%) using 2, 4-D combined with BAP. The callus induced during the current study did not form embryos when subcultured on GA_3 supplemented medium and hormone free-medium. Bronsema et al. (1997) made similar observation while working with maize. These observations concurs to those of Danso (1997), who reported in cassava that root development occurred when calli derived from *in vitro* explants were transferred to the embryos induction medium. Some attempts have been carried out in certain species to associate the endogenous hormone levels of explants and cultures derived from them with their regeneration competence (Sasaki et al., 1994; Hess and Carman, 1998; Jiménez and Bangerth, 2001). The effect of any particular exogenously applied growth hormone is influenced by a variety of other factors in the internal environment of the plant, especially other hormones in the plant (Onwubiku, 2007; Preece, 1987). The fact that the induced calli were not able to produced embryos in current study could indicate that the endogenous levels of both cytokinins and auxins in these cassava varieties might be adequate. Hence, exogenous application led to supra-optimal amounts which may induce some inhibitory effects.

Effect of growth regulators on the CMD2 conformity in of the induced calli

During the current study, it was possible to isolate somaclonal variants through callus phase based on the CMD2 resistant gene in cassava. SSR primer revealed the loss of the resistant gene (319 bp) in callus induced from 2, 4-D media in cultivars agric-rouge and agblehoundo and in callus induced from TDZ medium in agblehoundo. These results concur with those of Beyene et al. (2016) and Chauhan et al. (2018) who reported the loss of CMD2 resistant gene after regenerating the entire cassava plants. Findings confirm the usefulness of SSR markers in the analysis of conformity of induced calli, similar to works on crops such as caladium tissue culture-derived plants by Cao et al. (2016). Similarly, Sharma et al. (2015) in *Stevia rebaudiana* showed genetic variation among the mother plants and callus induced from 2,4-D medium. This study showed that the loss of CMD2 resistance gene at the callus stage is genotype depended since the 319 bp fragment was present callus induced from Atinwewe cultivar while it was absent in others. This is most apparent in crops under *in vitro* culture where the amount of growth regulator is extensive. On the other hand, SCAR primer showed the presence of the resistant fragment (700 bp) in the induced calli from both 2, 4-D and TDZ media in cultivar agblehoundo which was similar to their mother. As in this study, SCAR marker was developed by Paran and Michelmore (1993) for downy mildew resistance genes in lettuce. SCARs may identify polymorphisms that are less accessible by other techniques. The efficiency of SCAR marker in the current study has been proved for authentication of traits in various biological systems vis-à-vis *Sorghum halepense* by Zhang et al. (2013) and in *Pennisetum glaucum* by Jogaiah et al. (2014). It was then concluded that the used of exogenous hormones such as 2, 4-D and TDZ caused the loss of the resistant fragments (319 bp) alone in the induced callus from Atinwewe cultivar while the second fragment was retained. Furthermore, the protocol established here may be of interest to detect and eliminate variants at early stages to minimize loss later after regenerating the whole plant (Radhakrishnan and Ranjitha, 2008; Roels et al., 2005). This preliminary study may open up new perspectives for implementing a biotechnological genetic improvement program for these cassava cultivars to other diseases.

Conclusion

In conclusion, the optimum Jik (3.85% NaOCl) concentration for sterilization of leaf explants was established to be 2% exposed for 15 min. The growth regulator 2,4-dichlorophenoxy acetic acid (2, 4-D) proved to be the best for callus formation with the optimum concentration of 2 µM for all cultivars. Furthermore, according to the cultivars, SSR and SCARS primers revealed either presence or absence of the CMD2 resistance gene in the induced calli compared to the mother plant.

Recommendation

Further investigation should be carried out to establish the optimal growth regulators for regenerating plantlets from the callus induced from the leaf explants of the three elite cassava cultivars.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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