African Journal of Biotechnology

Volume 15 Number 29, 20 July 2016

ISSN 1684-5315

Academic Journals

ABOUT AJB

The African Journal of Biotechnology (AJB) (ISSN 1684-5315) is published weekly (one volume per year) by Academic Journals.

African Journal of Biotechnology (AJB), a new broad-based journal, is an open access journal that was founded on two key tenets: To publish the most exciting research in all areas of applied biochemistry, industrial microbiology, molecular biology, genomics and proteomics, food and agricultural technologies, and metabolic engineering. Secondly, to provide the most rapid turn-around time possible for reviewing and publishing, and to disseminate the articles freely for teaching and reference purposes. All articles published in AJB are peer-reviewed.

Contact Us

Editorial Office: ajb@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJB

Submit manuscript online http://ms.academicjournals.me/

Editorial Board

African Journal of Biotechnology

Table of Content: Volume 15 Number 29 20 July, 2016

ARTICLES

academicJournals

Vol. 15(29), pp. 1531-1540, 20 July, 2016
DOI: 10.5897/AJB2016.15453
Article Number: BD0FD0659627
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Effect of carbohydrate source on the *in vitro* germination of *Elaeis guineensis* Jacq. zygotic embryos on two basal media

Okafor, Uche Cyprian* and Okezie, Carl Eziuche Agaba

Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, Enugu State, Nigeria.

Received 4 May 2016, Accepted 11 July, 2016.

In this study, seven carbohydrates, namely, glucose, fructose, galactose, sucrose, maltose, raffinose and starch were screened for the most suitable carbon source for the *in vitro* culture of oil palm (*Elaeis guineensis* Jacq. var. tenera) zygotic embryos on the basal media of Murashige and Skoog (MS) (1962) and Eeuwen (Y3) (1976). The embryos were excised aseptically and cultured on both media containing the different carbohydrates. Results obtained showed that while both media supported *in vitro* plantlet regeneration from the embryo explants, Y3 medium was significantly superior to MS medium (P=0.05) in length of root and shoot of plantlets produced. Sucrose was also significantly (P=0.05) superior to glucose and maltose while starch, raffinose, fructose and galactose had the least value in all growth parameters studied. In addition, more uniform plantlets were produced in Y3 media in which sucrose served as carbon source relative to the other six carbon sources tested. The protocol reported here has potential for speeding up germination process within a short period of time for oil palm.

Key words: *Elaeis guineensis,* Eeuwen (Y3) medium, Murashige and Skoog (MS) (1962) medium, embryo explants, carbohydrates.

INTRODUCTION

Oil palm, a member of the family Arecaceae, genus *Elaeis*, has two known species namely, *Elaeis guineensis* (African oil palm) and *Elaeis oleifera* (American oil palm). Oil palm is an arborescent, monocotyledon and an oleaginous tropical perennial crop, used as vegetable oil as well as a substitute for biofuel (a renewable energy source of energy) (Marlucia et al., 2014). There are three varieties of oil palm classified based on the presence or absence of a shell in the fruit: Dura which produces fruits with a thick shell; Pisifera which is without a shell in its rare fruits; Tenera, a hybrid of these two varieties and produces fruits with an intermediate shell (Kamnoon and Preamrudee, 1999).

Oil palm breeding by conventional methods has several limitations which are, (i) the perennial nature of the plant (which may require about five to ten years to assess the value of a progeny); (ii) the allogamous nature and, (iii) the use of seeds as the sole means of propagation

*Corresponding author. Email: uche.okafor1287@unn.edu.ng.

Figure 1a. Seeds of *Elaeis guineensis* Jacq. (Mag. 1x).

material (Corley, 1982). For these reasons, *in vitro* propagation bridges the gap and offers solutions to such possible problems.

Plant tissue culture techniques and in particular embryo culture ensures rapid *in vitro* regeneration of plantlets as well as shortening of germination time (Pullaiah and Subba-Rao, 2009; Marlucia et al., 2014). Previous reports by Wan Nur Syuhada et al. (2016) (successfully used immature embryos for plantlet regeneration of oil palm through indirect organogenesis); Kantamaht et al. (2010), Alves et al. (2011), Suranthran et al. (2011) and Thuzar et al. (2012) also achieved maximum results on *E. guinensis* embryo-derived callus, hybrids, Dura and Tenera varieties, respectively when the carbon sources used were at three percent w/v. This appears to justify the concentration of carbon source used in this work even though MS was the only basal medium used by the authors cited. Muniran et al. (2008) also achieved good results when carbon sources used were at three percent even though they compared the effect of three basal media on *E. guineensis* var. Dura. Subsequently, the carbon sources also used did not include a trisaccharide and a polysaccharide. The present work, however, compared two basal media (MS and Y3), used seven carbon sources which comprises monosaccharides (glucose, galactose and fructose), disaccharides (sucrose and maltose), a trisaccharide (raffinose) and a polysaccharide (starch).

Carbohydrates are required *in vitro* due to the heterotrophic nature of cultured cells, to replace the carbon, which plants normally fix from the atmosphere by photosynthesis *in vivo* for growth, development and other physiological processes (Mehwish et al., 2013) Carbohydrates primarily contain only carbon, hydrogen and oxygen (Noggle and Fritz, 2006). In plants, they serve as the principal vehicle in which the energy of sunlight is captured and they make up their structural scaffold (Datta, 2007). Many explants in culture are generally not autotrophic and so require a carbon source that plays an important role as an energy source, as well as an osmotic agent (Smith, 2013). Thus, they are necessary as a source of energy and a carbon substrate for biosynthesis and its continuous supply to plants cultured *in vitro* is essential, since photosynthetic activity of *in vitro* grown tissues is usually reduced. For all these reasons, sugars have a great potential effect on the physiology, growth and differentiation of cells (Gibson, 2000). Carbohydrates may be divided into large groups namely; monosaccharides (glucose, fructose, galactose, etc.), oligosaccharides which comprised of disaccharides (sucrose, maltose, etc.) and trisaccharides (raffinose, etc.) and polysaccharides (starch, etc.) (Datta, 2007). Hilae and Te-chato (2005) investigated the effect of different carbon sources and strengths of MS medium on oil palm somatic embryo induction of shoot and root from haustorium-stage embryos. A reduced strength of MS and high concentration of alcohol sugar or sucrose was found to enhance root formation. Boonsanong and Kamnoon (1996) reported the superiority of Y3 or ½ MS when compared with MS in the formation of developed growth of root from zygotic embryos. In addition, Muniran et al. (2008) compared the efficacy of three basal media: N_6, MS and Y3 in the micropropagation of oil palm. The superiority of Y3 in callus induction, somatic embryogenesis and rooting was observed as compared to MS (Marlucia et al., 2014). Also, a combination of both MS and Y3 as reported by Wan Nur Syuhada et al. (2016) showed highest number of friable callus when immature embryo of oil palm is used as the explant. This study was therefore carried out to employ two types of modified basal media, namely, Murashige and Skoog (1962) and Eeuwen (1976), and to use a wide range of carbon sources (glucose, fructose, galactose, sucrose, maltose, raffinose and starch) as energy sources for purposes of determining the most suitable for studying the growth and development of oil palm embryos.

MATERIALS AND METHODS

Site of experiment

This study was conducted at the Tissue Culture and Molecular Biology Laboratory of the National Biotechnology Development Agency (NABDA) located at University of Nigeria, Nsukka.

Source of explants

The mature zygotic embryos employed in this study were excised from mature seeds of *E. guineensis* Jacq. 'tenera hybrid' obtained from the Nigerian Institute for Oil Palm Research (NIFOR), Benin City, Edo State, Nigeria. The seeds were identified and confirmed at the herbarium of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. Oil palm seeds (Figure 1a) were cracked with a hammer to extract the embryo after removing the pericarp from the fruit. Prior to extracting the embryo (Figure 1b), the seeds were immersed in a mild solution of water

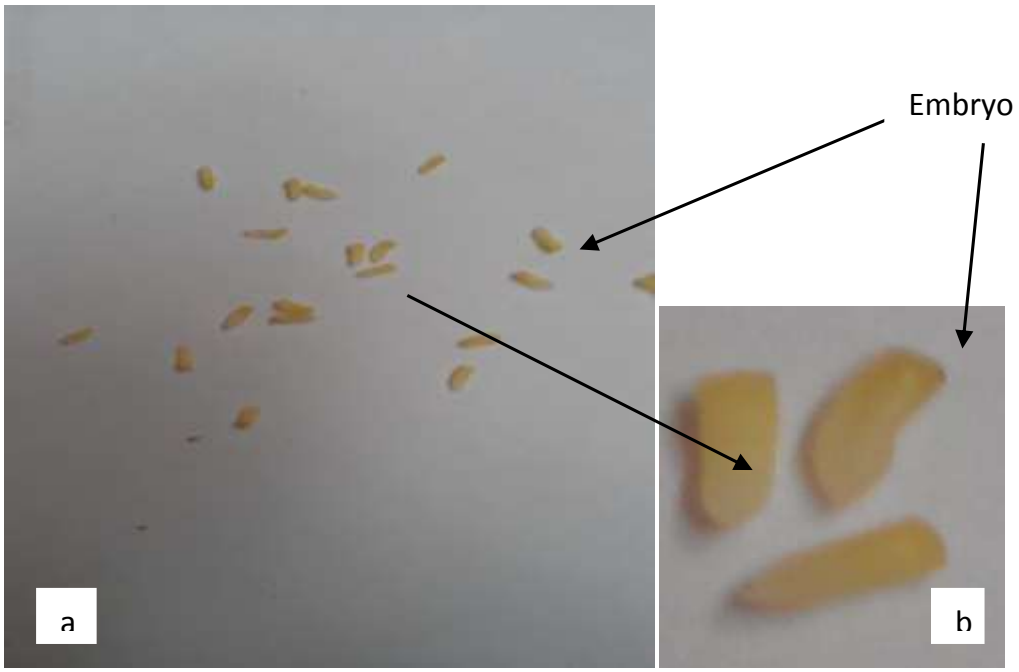


Figure 1b. i: Oil palm zygotic embryos (light yellow in colour) measuring between 0.9-1.2 cm in length (Mag 1x) ii: enlarged oil palm zygotic embryo (Mag. 3x).

and Tween-20 and were swirled for one minute according to the method of Suranthran et al. (2011).

Culture media

The basal culture media employed in this study were those of Murashige and Skoog (1962) and Eeuwen (1976) (Table 1). Each was supplemented with 3% (w/v) of the carbohydrates, namely, glucose, fructose, galactose, sucrose, maltose, raffinose and starch. Cultures with no carbohydrate source were employed as controls.

Sterilization techniques and embryo transfer

The seeds (Figure 1a) were washed in running tap water to remove visible dirt and other adhering particles and then surface-sterilized by immersion in seventy percent ethanol (v/v) for five seconds and subsequently in one percent (v/v) sodium hypochlorite (NaOCl) from commercial bleach (Clorox) for ten minutes, followed by four rinses in sterile double distilled water. Prior to inoculation on the growth media, the seeds were freed of the seed coats and endosperm exposing the embryo (Figure 1b), using a pair of sterile forceps and scalpels, on a 9 cm-diameter Whatman No. 1 filter paper in a Petri dish. The seed testa and endosperm were discarded after every manipulation to minimize contamination. Embryos were transferred singly into culture vessels (one per vessel). In the course of embryo transfer, the pair of forceps was dipped in 70% ethanol, flamed over a spirit lamp, and held to cool within the sterile hood before picking up the embryos into the culture media. Similarly, the mouth of the inoculated culture vessel was flamed over a spirit lamp before closing. These were done to minimize contamination. The cultured explants were maintained in the growth room at 25 ±2°C under 16-h light/8-h dark cycles at a light intensity of 2500 lux provided by cool white fluorescent tubes (Quickstart, B.G. Tropical Daylight 6,500k, England). The process of surface-sterilization of seeds and the inoculation of embryos were all carried out in a laminar air-flow hood, previously made sterile by swabbing with absolute ethanol and exposed to ultraviolet light for 30 min.

Experimental design and statistical analysis

The experiment was set up as a completely randomized two-factor factorial design with the main factors being basal media salt formulation at two levels (MS and Y3 media) and carbon sources at seven levels (glucose, fructose, galactose, sucrose, maltose, raffinose and starch) plus control, making it a total of eight levels. A total of 480 embryos (240 for each of the basal medium) were used for this experiment, that is, ten replicates (each replication consisted of one embryo per culture tube) for eight treatments, done three independent times, and used in order to obtain a mean ± standard error for all the growth parameters studied. Data pertaining to growth parameters which included: Leaf area, fresh weight, length of roots and length of shoots was also subjected to analysis of variance (ANOVA) by the selection of ten plantlets. Treatment means were tested for significance ($P \leq 0.05$) using Duncan mutiple range test (DMRT) error bars were represented at 5% value.

Plant regeneration studies

The growth and development of embryos were monitored on daily basis from the day of inoculation. Time course in sprouting (%) and sprouting rate were determined from the first day till the fourteenth day when sprouting had leveled off in all the treatments. At the end of eighth week, regenerated plantlets under each treatment were withdrawn from the culture media and scored for the following growth parameters: length of shoots and roots produced, fresh weight of sprouts produced (weighed on a Sartorius sensitive

Table 1. Compositions of Murashige and Skoog (1962) and Eeuwens (1976) basal media.

Component	Y3	MS
Macroelements (mg/l)		
KNO_3	2020	1900
KCl	1492	-
KH_2PO_4	-	170
NH_4Cl	535	-
NH_4NO_3	-	1650
$NaH_2PO_4.2H_2O$	312	-
$CaCl_2.2H_2O$	294	440
$MgSO_4.7H_2O$	247	370
Iron salts (mg/l)		
$Na_2EDTA.2H_2O$	37.3	37.3
$FeSO_4.7H_2O$	27.8	27.8
Microelements (mg/l)		
$MnSO_4.4H_2O$	11.2	22.3
KI	8.3	0.83
$ZnSO_4.7H_2O$	7.2	8.6
H_3BO_3	3.1	6.2
$CoCl_2.6H_2O$	0.24	-
$Na_2MoO_4.2H_2O$	0.24	0.25
$CuSO_4.5H_2O$	0.16	0.025
$NiCl_2.6H_2O$	0.024	-
Supplements (mg/l)		
Meso inositol	100	100
Thiamine-HCl	0.5	0.1
Pyridoxine-HC	0.05	0.5
Calcium pentothenate	0.05	-
Nicotinic acid	0.05	0.5
Biotin (B-complex)	0.05	-
Gibberelic acid	0.038	-
Glycine	-	2
Sucrose	45000	30000
Coconut water	5%	-
Activated charcoal	1000	-
Agar (g/l)	7.5	7.5
pH	5.5	5.8

weighing balance Sartorius BS 323S, USA) and plantlet leaf area. The experiment was repeated three independent times for reproducibility.

RESULTS

The pattern of oil palm zygotic embryo development and morphogenesis during the first ten days of culture is shown in Figure 1c (i-iv). Swelling and expansion of embryos was observed within the first 3 days (Figure 1c-iii) and it was followed by the curving of plantlets approximately 3 to 5 days of culture (Figure 1c-iv). The embryo (Figure 1b) which was light yellow in colour at the time of inoculation enlarged and began to turn green leading to the emergence of radicle from the radicular end and plumule from the plumular end and a haustorium within 5 days in culture. The radicle and plumule finally gave rise to the root and new shoot, respectively, while the haustorium served an absorptive function (Figure 1c-vi).

Sprouting commenced on the third day in culture. Sprouting in both MS and Y3 basal media supplemented with sucrose had 50% sprouting on the 5^{th} day and maximum sprouting percentage of 81.2±2.58 and 68.00±2.99 on the 14^{th} day for Y3 and MS basal media, respectively. For glucose, 50% sprouting in both MS and Y3 basal media was achieved on the 6^{th} and 5^{th} day, with a maximum of 60.40±3.84 and 58.40±2.01 on the 14^{th} day, respectively. Maltose, however, had 50% sprouting on the 12^{th} and 7^{th} day and maximum percent sprouting of 50.00±1.84 and 51.40±3.72 on the 14^{th} day for MS and Y3 basal media, respectively. There was no sprouting in those embryo explants supplemented with fructose, galactose, raffinose, starch and control within 14 days in culture (Table 2).

Sprout rate of the plantlets (determined as the reciprocal of the number of days to 50% sprouting) was dependent on both the type of carbon source employed and basal media used. The highest rate of sprouting (0.20±0.01) was recorded for Y3+sucrose, Y3+glucose and MS+sucrose each since 50% sprouting was achieved on the 5^{th} day, while MS+glucose had 0.16±0.05 because 50% sprouting was achieved on the 6^{th} day while maltose in both Y3 and MS had sprout rates of 0.14±0.00 and 0.08±0.00, respectively, because 50% sprouting was achieved on the 7^{th} and 12^{th} day, respectively. Embryo explants in untreated (control), fructose, galactose, raffinose and starch in both media did not sprout and a value of 0.00±0.00 was recorded for them (Table 3).

One-way analysis of variance (ANOVA) showed that Y3 and MS media were significant at 5% level in all growth parameters studied except for fresh weight and leaf area. The two media brought about *in vitro* plantlet regeneration from embryo of *E. guineensis*. The media had significant effects on the length of root and shoot of the plantlets. The lengths of roots (Figure 2) and shoots (Figure 3) produced by the plantlets, achieved on Y3 medium were 4.18 ± 1.29 and 1.08 ± 0.54 cm; and 3.84 ± 0.18; 2.98± 0.73 cm for sucrose and glucose, respectively, while the same parameters achieved on MS medium recorded 2.08 ± 0.77, 0.68 ± 0.25; and 3.34 ± 0.86; 2.00±0.84 cm for sucrose and glucose, respectively. This may suggest in this study that, Y3 medium was found superior to MS medium for *in vitro* regeneration of *E. guineensis* through embryo culture in

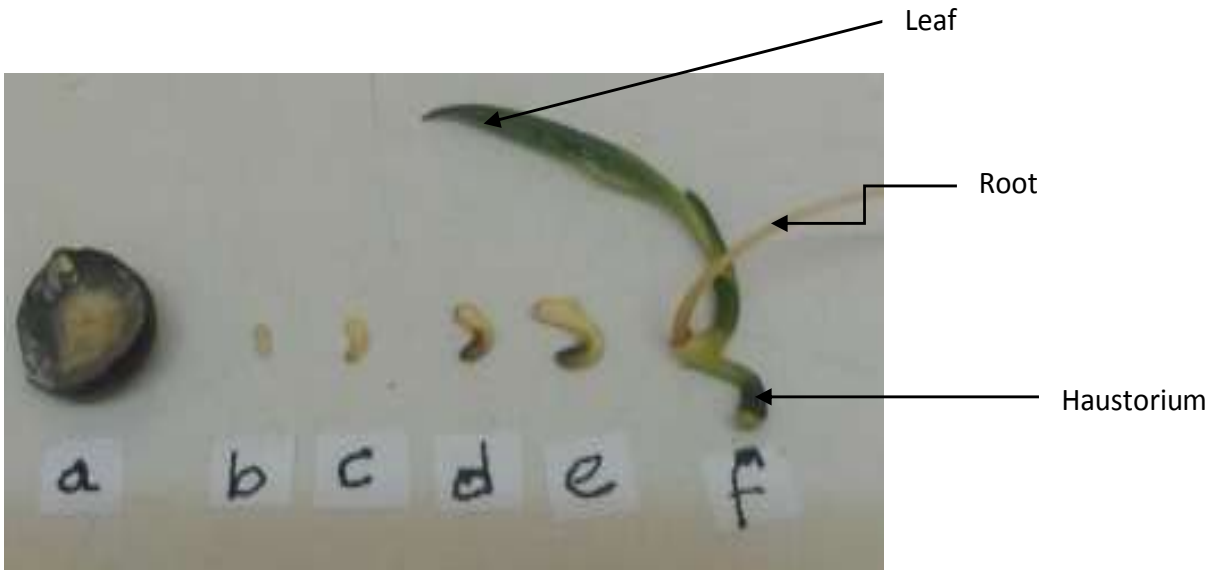


Figure 1c. Pattern of development of the plantlets from zygotic embryo of oil palm supplemented with sucrose on Y3 medium. i: Longitudinal section of oil palm seed showing the embryo, endosperm and testa (Mag. 1x); ii: Excised zygotic embryo (Mag. 0.5x); iii: Curved zygotic embryo after three days in culture (Mag. 0.5x); iv: Curved zygotic embryo between four and ten days in culture (Mag. 0.25x); v: Swollen and curved zygotic embryo after fourteen days in culture (Mag. 0.5x); vi: Plantlet after eight weeks in culture showing leaf, root and haustorium (Mag. 1x).

Table 2. Sprouting percentage of *Elaeis guineensis* embryo explants after two weeks on Y3 and MS basal media containing different carbon sources at three % (w/v) concentration.

Carbon sources	Media	
	MS	Y3
Sucrose	68.00±2.99^{a1}	81.20±2.58^{a2}
Glucose	60.40±3.84^{b1}	58.4±2.01^{b1}
Maltose	50.00±1.84^{c1}	51.4±3.72^{c1}
Fructose	0.00±0.00^{d1}	0.00±0.00^{d1}
Galactose	0.00±0.00^{d1}	0.00±0.00^{d1}
Raffinose	0.00±0.00^{d1}	0.00±0.00^{d1}
Starch	0.00±0.00^{d1}	0.00±0.00^{d1}
Control	0.00±0.00^{d1}	0.00±0.00^{d1}

Means followed by the same letters do not differ, in the column, at 5% level of probability using Duncan's multiple range test and means followed by the same number do not differ, in the row at 5% level of probability using DMRT.

Table 3. Rate of sprouting as affected by the various carbon sources at three percent (w/v) and the basal media.

Carbon sources	Media	
	MS	Y3
Sucrose	0.20±0.01^{a1}	0.20±0.01^{a1}
Glucose	0.16±0.05^{a1}	0.20±0.01^{a1}
Maltose	0.08±0.00^{b1}	0.14±0.00^{b2}
Fructose	0.00±0.00^{c1}	0.00±0.00^{c1}
Galactose	0.00±0.00^{c1}	0.00±0.00^{c1}
Raffinose	0.00±0.00^{c1}	0.00±0.00^{c1}
Starch	0.00±0.00^{c1}	0.00±0.00^{c1}
Control	0.00±0.00^{c1}	0.00±0.00^{c1}

Means followed by the same letters do not differ, in the column, at 5% level of probability using Duncan's multiple range test and means followed by the same numbers do not differ, in the row at 5% level of probability using DMRT.

the length of root and shoot. Explants in Y3 medium supplemented with sucrose had the highest fresh weight of 1.77 ± 0.48 g while the least root length of 0.04 ± 0.02 g was recorded for the explants in galactose+Y3 (Figure 4). ANOVA showed that there was significant difference in carbon sources used, but significant difference did not exist between MS and Y3 under each carbon source. This means that the use carbon source irrespective of basal media used had a significant effect on the fresh weight of sprouts. In Y3 medium, explants supplemented with sucrose had the highest leaf area of 6.70 ± 0.91 cm^2 as compared to glucose while there was no leaf for the untreated explants (control), fructose, galactose, maltose, raffinose and those supplemented with starch, thus, 0.00 ± 0.00 cm^2 was recorded for them (Figure 5). ANOVA showed that there was no significant difference in basal media but sucrose was a better carbon source than glucose. This means that only the application of carbon source had a significant effect on the leaf area of

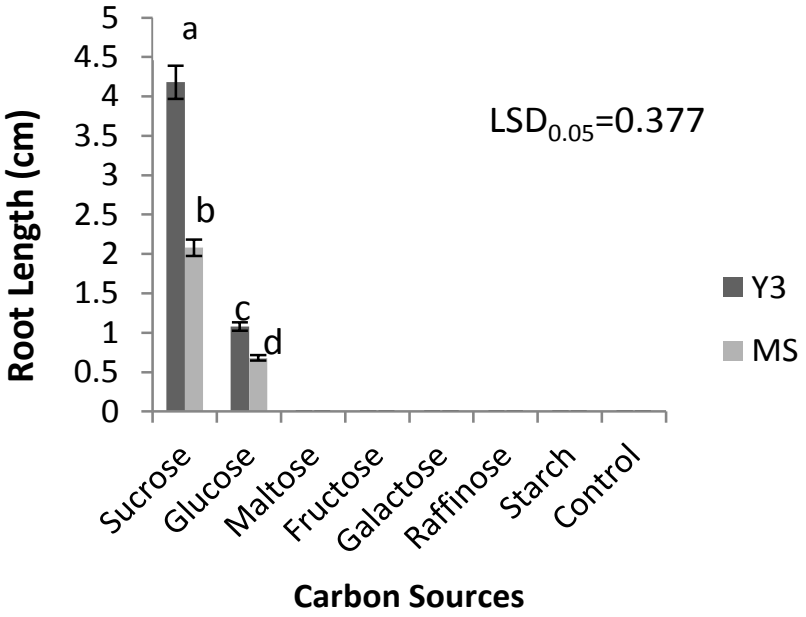


Figure 2. The effects of different carbon sources and basal media on the root length of plantlets of *E. guineensis* (Jacq.) produced in MS and Y3 media. Each point represents a mean of ten replicate treatments. Vertical bars represent ± standard error of the mean. Means followed by the same letters from one column to another do not differ at 5% level of probability using Duncan's multiple range test.

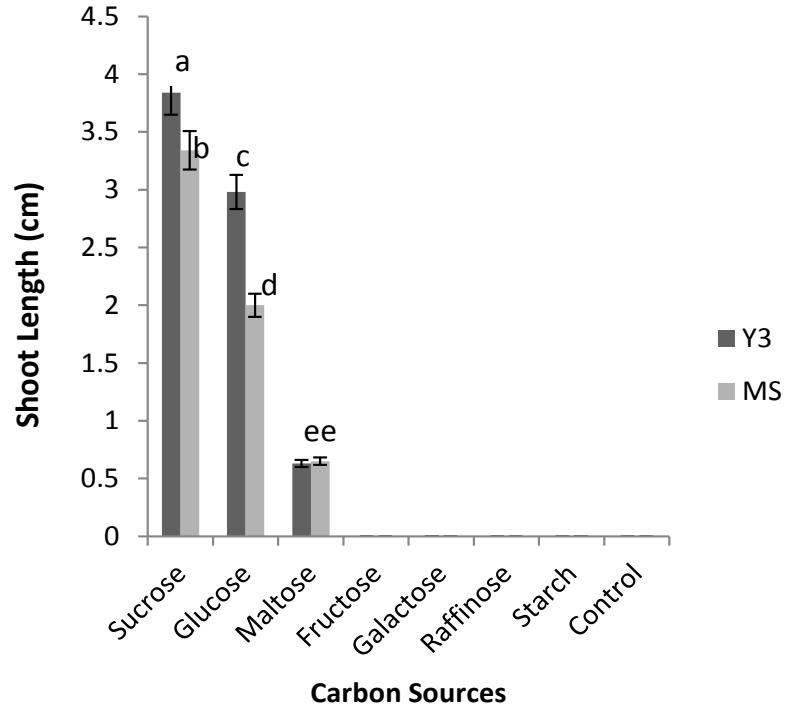


Figure 3. The effects of different carbon sources and basal media on the shoot length of plantlets of *Elaeis guineensis* (Jacq.) produced in MS and Y3 media. Each point represents a mean of ten replicate treatments. Vertical bars represent ± standard error of the mean. Means followed by the same letters from one column to another do not differ at 5% level of probability using Duncan's multiple range test.

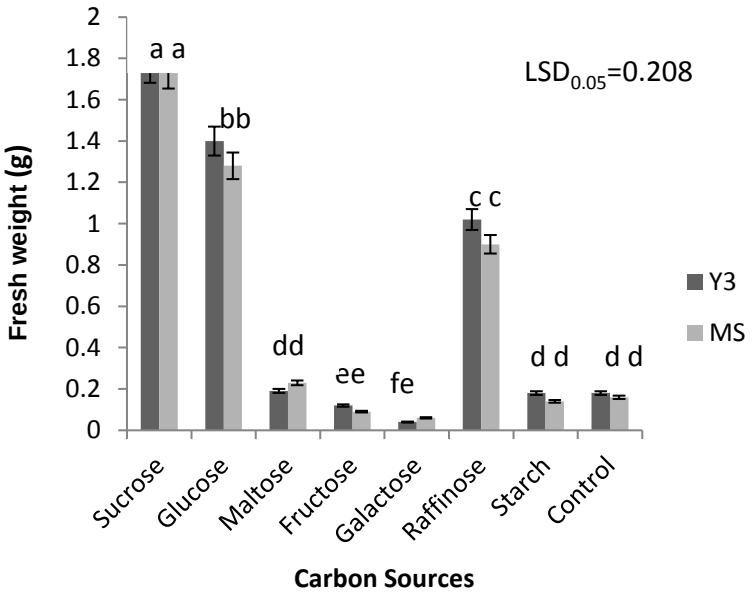


Figure 4. The effects of different carbon sources and basal media on the fresh weight of plantlets of *Elaeis guineensis* (Jacq.) produced in MS and Y3 media. Each point represents a mean of ten replicate treatments. Vertical bars represent ± standard error of the mean. Means followed by the same letters from one column to another do not differ at 5% level of probability using Duncan's multiple range test.

plantlets.

DISCUSSION

The addition of carbon sources to *in vitro* cultures is necessary prior to autotrophy as seen in this study. The embryonic axis that turned from yellow to green about three to five days from the time of inoculation indicate that the explants had become autotrophic. Similar result was obtained by Suranthran et al. (2011) who observed swelling, expansion and greening of embryos within five days of culture. The authors observed plumule emergence which eventually led to the emergence of shoot from the shoot apex within fourteen days of culture. The control (devoid of carbon source) did not show any marked differentiation into plumule and radical, an indication that sugars are mandatory for morphogenesis.

Sucrose was superior to other carbon sources employed in all growth parameters studied, producing healthy and uniform plantlet (Figure 1d). The reason may be that it is the most common carbohydrate translocated in the phloem sap of many plants (George, 2008). In addition, sucrose, a non-reducing sugar with no free carbonyl group has all its aldehydes (the reactive part of the sugars) hidden, preventing them from undergoing glycosylations that could kill the plant. This, however, makes sucrose very stable and convenient to transport sugars (David and Michael, 2008). The presence of the hydrolytic enzyme 'invertase' in plant cell wall and vacuole that breaks down sucrose into glucose and fructose for plant metabolism may be responsible for the superiority of sucrose over other carbon sources (Arnd and Guo-Qing, 1999). The superiority of sucrose over the other sugars employed as carbon source in this study is consistent with earlier reports for other plants such as *Phoenix dactylifera* (Veramendi and Navarro, 1996; Othmani et al., 2009) used as a sole carbon source and *Dianthus caryophyllus* (Karami et al., 2006). Baskaran and Jayabalan (2005) suggested that among the three carbon sources, sucrose proved to be better than fructose or glucose for shoot regeneration of *Eclipta alba*. Glucose, on the other hand supported *in vitro* plantlet regeneration of zygotic embryos of oil palm, although, produced lesser root, shoot, leaf area and fresh weight as compared to sucrose. This may be as a result of a series of chemical reactions (glycosylation) that could inhibit growth since glucose is a reducing sugar (with a free carbonyl group). In contrast to the results obtained, Amiri and Kazemitabar (2011) suggested that glucose was better for inducing shoot proliferation than other carbon sources in *Datura stramonium***.**.

Maltose, a reducing sugar favored the formation of plumule that resulted in the production of shoot with neither leaves nor root when Y3 was used (Figure 1e). This may have resulted to the slow hydrolysis of maltose by *in vitro* plantlet as reported by George (2008). The result obtained in this study is consistent with that of

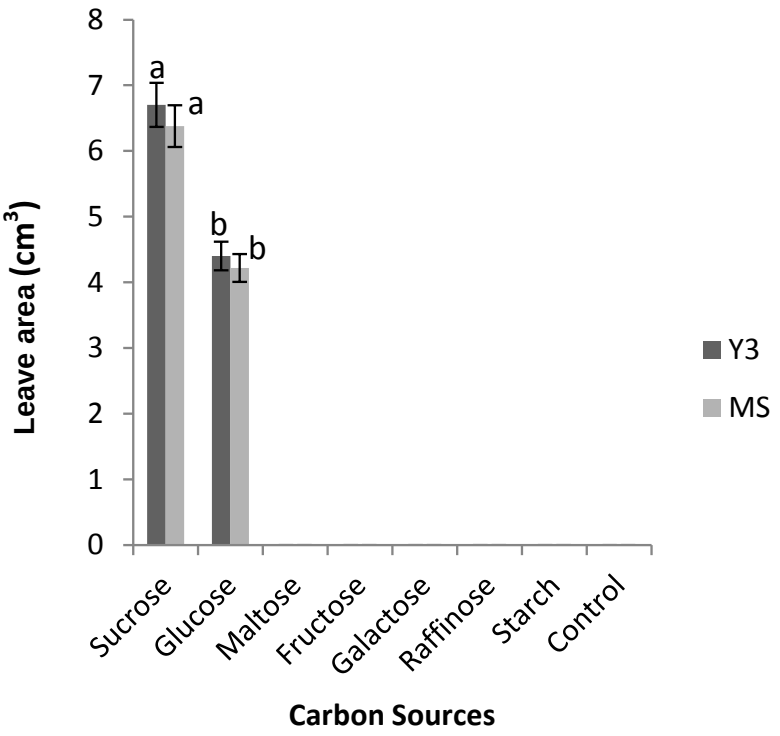


Figure 5. The effects of different carbon sources and basal media on the leaf area of plantlets of *Elaeis guineensis* (Jacq.) produced in MS and Y3 media. Each point represents a mean of ten replicate treatments. Vertical bars represent ± standard error of the mean. Means followed by the same letters from one column to another do not differ at 5% level of probability using Duncan's multiple range test.

Hossain et al. (2005). The authors reported that maltose is inferior to sucrose and glucose in the production of root in *Centella asiatica*. This could be attributed to the fact that plants may not have the necessary enzyme 'maltase' to hydrolyse maltose into glucose. These, however, may have also resulted to low rate of sprouting seen in the results obtained.

The inability of the embryos to utilize the more complex carbohydrate (starch) may have been as a result of the absence of the full complements of enzymes needed for their degradation or conversion into simpler utilizable carbohydrates such as sucrose and glucose. Raffinose, on the other hand could not support embryo growth in both media possibly due to the presence of galactose (a product in the hydrolysis of raffinose). The embryo explants elongated but could not be differentiated into plumule and radicle (Figure 1f). This may be as a result of the lack of galactose kinase which reduces the toxicity of galactose by converting it to galactose-1-phosphate as found by George (2008) who reported the toxicity of galactose in plant tissues of orchids. This, however, may be the same reason the embryo explants of *E. guineensis* could not sprout when raffinose was employed as the carbon source. In addition, the elongation resulted because sucrose, a product of the hydrolysis of raffinose may have been taken up by the embryo explants but could not differentiate because galactose may have inhibited the growth resulting in increase in fresh weight especially on Y3 medium. Mehwish et al. (2013) reported absence of roots when xylose, lactose, turanose, raffinose, cellulose, starch or mannitol were employed as carbon source for the propagation of *Chrysanthemum* both in light and dark conditions.

Fructose, on the other hand did not support the zygotic embryo regeneration of *E. guineensis* possibly due to the production of furfural derivatives by fructose during autoclaving as reported by George (2008). This may be toxic to the explants and may have caused the failure of embryos to sprout when fructose or galacose was used. The superiority of Y3 to MS could be related to the quantity of ions in the basal medium. Bhojwani and Razdan (1996) showed that the main difference in the composition of a range of commonly used tissue culture media is based on the quantity of various salts and ions. Plantlets in Y3 medium irrespective of the carbon source employed showed marked difference in their root growth as compared to the ones in MS medium. This observation may have been attributed to the high nitrogen content of MS that may have affected root organogenesis of *E. guineensis* plantlets. Wan and

Schuyler (2004) supported this by reporting that reducing the nitrogen content in the MS medium by halving could alleviate the problem of toxicity, and thus resulting in a high frequency of shoot organogenesis in *Pinus pinea* L.

In addition, the compositions of macro and micro elements in Y3 media has been reported to be more suitable for palm species as compared to White medium, or Murashige and Skoog (1962) and Eeuwens (1976). The plantlet regenerated on Y3 medium showed profuse longer roots when compared with MS, this could be attributed to the content of the medium. Eeuwens medium contains higher concentration of KCl and NH_4Cl, which provides more Cl^- ions.

The Cl^- ions are known to act like natural auxins in the somatic embryogenesis, rooting and callus induction of oil palm immature embryos (Muniran et al., 2008, Masani et al., 2013). In addition, Y3 medium is richer in micro salt content (KI), which might play a vital role in the root induction. Boonsanong and Kamnoon (1996) also confirmed the superiority of Y3 over MS in root development and in general growth of oil palm zygotic embryos. Marlucia et al. (2014) also reported that Y3 medium generates better oil palm plantlets for hardening when a 90-day old embryo was used as explant. The zygotic embryos of *E. guineensis* (Jacq.) used in this study showed different responses to carbon sources and basal medium. Eeuwen medium supplemented with sucrose (3%) was noted to favour the growth of the embryos *in vitro* by increasing the growth of plantlets especially in their fresh weights and long profuse roots as compared to other carbon sources in both media.

Conclusion

The micropropagation protocols for *E. guineensis* Jacq. as described in this study have the potential for shortening the germination time required to obtain *E. guineensis* plantlets when compared with normal soil germination. The plantlets, after hardening, would be raised *ex vitro* to ensure a steady supply of vegetable oil from the seeds. Somaclonal variants arising from this propagation method (Bhojwani and Razdan, 1996) would include those with desirable attributes that would enhance steady availability of elite cultivars of this plant for purposes of providing a cleaner and more environmentally friendly substitute for fossil fuel and a source of commercial vegetable oil.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Alves SAO, De- lemos FO, Filho BGD, Da silva ALL (2011). *In vitro* protocol optimization for development of interspecific hybrids of oil palm (*Elaeis oleifera* Cortés x *Elaeis guineensis* Jacq.) J. Biotechnol. Biodivers. 2(3):1-6.

Amiri S, Kazemitabar SK (2011). Enhancement of callus induction and regeneration efficiency from embryo cultures of *Datura stramonium* by adjusting carbon sources and concentrations. Afr. J. Biotechnol. 10(50):10101-10107.

Arnd S, Guo-Qing T (1999). The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning, Trends Plant Sci. 4(10):401-407.

Baskaran P, Jayabalan N (2005). Role of basal media, carbon sources and growth regulators in micropropagation of *Eclipta alba* – a valuable medicinal herb. Kmitl. Sci. J. 5(2):469-482.

Bhojwani SS, Razdan MK (1996). Plant Tissue Culture: Theory and Practice, a Revised Edition. Elsevier, Netherlands, P 779.

Boonsanong C, Kamnoon K (1996). *In vitro* culture of embryos and callus of oil palm (*Elaeis guineensis Jacq*.) J. Sci. Soc. Thailand 22:1-12.

Corley RHV (1982). Clonal planting material for the oil palm industry. Planter 58:515-528.

David LN, Michael MC (2008). Lehninger Principles of Biochemistry (5th ed.). Freeman WH and Company, New York, P 1302.

Datta SC (2007). *Plant Physiology*. New Age International Publishers, New Delhi P 618.

Eeuwens CJ (1976). Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palms (*Cocos nucifera*) and cultured *in vitro*. Physiol. Plant. 36:23-28.

George EF (2008). Plant tissue culture procedure. In: George EF, Hall MA and Klerk GD (Eds.). Plant Propagation by Tissue Culture. Springer Publisher, Dordrecht, Netherlands, P 504.

Gibson SI (2000). Plant sugar-response pathways. Part of a complex regulatory web. Plant Physiol. 124:1532-1539.

Hilae A, Te-chato S (2005). Effects of carbon sources and strength of MS medium on germination of somatic embryos of oil palm (*Elaeis quineensis* Jacq.) Songklanakarin J. Sci. Technol. 27:629-635.

Hossain MA, Hossain MT, Ali MR, Rahman SMM (2005). Effect of different carbon sources on *in vitro* regeneration of Indian pennywort (*Centella asiatica* L.) Pak. J. Biol. Sci. 8(7):963-965.

Kamnoon K, Preamrudee D (1999). The origin and development of embryoids in oil palm (*Elaeis guineensis* Jacq.) embryo culture. Sci. Asia 25:195-202.

Kantamaht K, Amornrat P, Kamnoon K (2010). The Effect of Chitosan on the Organogenesis of Oil Palm Embryo-Derived Callus. Not. Bot. Horti Agrobo. 38(1):213-217.

Karami OA, Deljou M, Ostat-ahmadi P (2006). Effect of sucrose concentrations on somatic embryogenesis in carnation (*Dianthus caryophyllus* L.). Sci. Hortcult. 110:340-344.

Marlucia SP, Luciano VP, Marines FP, Luiz GTD, Evasto, MD, Vanessa CS (2014). Influence of culture medium and age of zygotic embryos on *in vitro* germination of *Elaeis guineensis* Jacq. Afr. J.Biotechnol. 13 (14):1515-1523.

Masani MYA, Noll G, Parveez GKA, Sambanthamurthi R, Prufer D (2013). Regeneration of viable oil palm plants from protoplasts by optimizing media components, growth regulators and cultivation procedures. Plant Sci. 210:118-127.

Mehwish Y, Touqeer A, Gaurav S, Alvaro S, Ishfaq AH (2013). Role of carbon sources for *in vitro* plant growth and development. Mol. Biol. Rep. 40:2837-2849.

Muniran F, Subhash JB, Farida HS (2008). Micropropagation of *Elaeis guineensis* Jacq. "Dura": Comparism of three basal media for efficient regeneration. Indian J. Exp. Biol. 46:79-82.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Noggle GR, Fritz GT (2006). Introductory Plant Physiology (2nd ed.). Prentice-Hall of India Private Limited, New Delhi, P 627.

Othmani A, Bayoudh C, Drira N, Marrakchi M, Tri M (2009). Somatic embryogenesis and plant regeneration in date palm Phœnix dactylifera L., cv. Boufeggous is significantly improved by fine chopping and partial desiccation of embryogenic callus Plant Cell Tiss. Organ Cult. 97:71-79.

Pullaiah T, Subba-Rao MV (2009). Plant Tissue Culture: Theory and Practice. Scientific Publishers, New Delhi, 197 p.

Smith RH (2013). Plant Tissue Culture Techniques and Experiments

(3rd ed.). Academic Press, San diego, P 188.
Suranthran P, Sinniah UR, Sreeramanan S, Maheran AA, Nordin R, Saikat G (2011). Effect of plant growth regulators and activated charcoal on *in vitro* growth and development of oil palm (*Elaeis guineensis* Jacq. var. Dura) zygotic embryo. Afr. J. Biotechnol. 10(52):10600-10606.
Thuzar M, Apichart V, Somvong T, Chatchawan J (2012). Recloning of regenerated plantlets from elite oil palm (*Elaeis guineensis* Jacq. var. Tenera). Afr. J. Biotechnol. 11(82):14761-14770.
Veramendi J, Navarro L (1996). Influence of physical conditions of nutrient medium and sucrose on somatic embryogenesis of date palm. Plant Cell Tissue Organ Cult. 45(2):159-164.
Wan Nur Syuhada WS, Rasid OA, Parveez GKA (2016). Evaluation on the effects of culture medium on regeneration of oil palm plantlets from immature embryos. J. Oil Palm Res. 28(2):234-239.
Whan S, Schuyler SK (2004). Effects of salt formulations, carbon sources, cytokinins, and auxin on shoot organogenesis from cotyledons of *Pinus pinea* L. Plant Growth Regul. 43:197-205.

academicJournals

Vol. 15(29), pp. 1541-1550, 20 July, 2016
DOI: 10.5897/AJB2015.14836
Article Number: BDA183159629
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Establishment of *in vitro* callus in sugarcane (*Saccharum officinarum* L.) varieties influenced by different auxins

Sardar Khatoon Solangi[1]*, Sadaf Tabasum Qureshi[1], Imtiaz Ahmed Khan[2] and Saboohi Raza[2]

[1]Institute of Plant Sciences, University of Sindh, Jamshoro, Pakistan.
[2]Nuclear Institute of Agriculture (NIA), Tandojam, Pakistan.

Received 4 July, 2015; Accepted 21 December, 2015

Sugarcane is an important perennial, polyploidy crop. Based on the growing demand, it has now attracted great attention as cash crop. Tissue culture technique, an alternative method for solving production problem and increasing production, was used in this study. The work was carried out at the Nuclear Institute of Agriculture (NIA), Tandojam, Pakistan. Standardization of protocol for proliferation of callus and induction of callus were established through *in vitro* culture using young meristem of sugarcane (*Saccharum officinarum* L.) as explants to enhance genetic variation in sugarcane varieties. Three varieties (NIA-2012, Gulabi-95 and NIA-105) were used. The shoot tips were supplemented with Murashige and Skoog (MS) medium modified with three auxins (2, 4-D, Picloram, NAA). All the auxins were applied in 0.0, 0.5, 1.0, 2.0 and 3.0 mg L^{-1}. MS basal medium was used as control free from concentration of auxins. Highly significant ($p < 0.05$) variations were observed in sugarcane varieties for all parameters of callus culture; while interactive effect of variety x treatment x concentration was non-significant for proliferation weight of callus. Among all the tested auxins 2, 4-D at 3.0 mgL^{-1} concentration proved to be the most effective auxin for callus proliferation and weight of all the sugarcane varieties. In light of the present research, it is concluded that auxins are preferable for future work in relation to *in vitro* callus induction for all varieties of sugarcane.

Key words: *Saccharum officinarum, in vitro,* callus induction, auxins, proliferation.

INTRODUCTION

Sugar cane (*Saccharum officinarum* L.) is an herbaceous agro industrial crop that belongs to the family Poaceae (Singh et al., 2003; Sharma, 2005; Cha-um et al., 2006). It is an important industrial crop of tropical and sub-tropical regions and is cultivated on 20 million hectares in more than 90 commercial countries because of its high trade value (Naz, 2003). Sugar juice is used for making sugar (Coax et al., 2000). Molasses (thick syrup residue) are used to produce ethanol (blended for motor fuel) and livestock feed. Bagasse (fibrous portion) is burned to provide heat and electricity for sugar mills, and green tops can be used as livestock feed (Mackintosh, 2000). It accounts for around 70% of the world's sugar (Khan et al., 2004). Sugarcane breeding programmes focus on the

*Corresponding author. E-mail: sardarkhatoon807@yahoo.com. Tel: 03470389956.

production of varieties with high yield, higher sucrose content, pest and disease resistance, tolerance to a biotic stress and improved rooting ability (Brumbley et al., 2008). The growing demand of newly released varieties could not be met by only conventional propagation methods as the multiplication rate set is 1:8. This restricts fast spread of superior varieties. Therefore, application of tissue culture techniques provides an alternative method for improvement of varieties (Sengar, 2011).

Tissue culture techniques have been widely used in *S. officinarum* L. for various purposes. Meristem tip or shoot tip culture has been used as a tool to produce virus-free plants (Hendre et al., 1975; Fitch et al., 2001; Tai and Miller 2001; Parmessur et al., 2002). Early efforts in sugarcane *in vitro* culture used a medium developed for efficient growth of meristem Tissue Culture150 (Thom et al., 1981; Lorenzo et al., 2001; Geijskes et al., 2003; Nieves et al., 2003; Wongkaew and Fletcher, 2004). These reports only focused on the effects of PGRs (plant growth regulators) such as BAP(6- benzylaminopurine), kinetin and coconut water on the MS medium for *in vitro* culture of commercial hybrid cultivars and *S. officinarum*. The chromosome number and ploidy levels in sugarcane plants have been reported by many authors. The type, concentration and combination of synthetic hormones of auxins caused somaclonal variation (Phillips et al., 1994). Tissue culture offers mass production (Czarinikow, 2010) of sugarcane. *In vitro* multiplication of sugarcane through callus culture, and shoot tip culture have been reported by many authors (Bakesha et al., 2002; Alam et al., 2003; Ali et al., 2008; Behara and Sahoo, 2009; Khan et al., 2012; Raza et al.,2014) to obtain regenerable type of callus. Tissue culture is efficient biotechnological tool for rapid multiplication of sugarcane plants (Kalunke et al., 2009; Kazim et al., 2015). It was also observed that callus derived from different auxins have different standardized protocols. Auxins are usually used to stimulate callus production and cell growth (Jahangir et al., 2009; Pandey et al., 2011; Shahid et al., 2012), to induce somatic embryogenesis (Edessoky et al., 2011), and stimulate growth of regenerable callus. In sugarcane tissue culture, no two genotypes give similar results within the culture conditions (Nahera et al., 1989; 1990; Smiullah et al., 2013; Mekonnen et al., 2014). This study aimed to develop genetic variability in sugarcane through callus culture, applying three auxins treatments with different concentrations. The effect of different growth regulator on the plant callus culture and extend of genetic variability induce by growth regulators among three sugarcane varieties are shown in this work.

MATERIALS AND METHODS

Explants source

Three elite hybrids of sugarcane NIA-2012 (early maturing), Gulabi-95 (mid maturing) and NIA-105 (late maturing) were used as explants source for callus culture.

Surface sterilization of explants

Apical meristem was chosen as source of explants because the cells are undifferentiated and meristematic cells actively divide. The most important reason is that there is no exposure to virus in the apical meristem and the production of virus free sugarcane explants is possible. The explants of sugarcane were taken in the laboratory. Unnecessary portion of the top was removed and the remaining was first washed with running tap water, and sterilized with 70% ethanol for 1 min and 10% sodium hypochlorite solution for 20 min (Figure 1). After sterilization, the explant materials were washed with double distilled sterilized water 2 to 3 times to remove any traces of disinfectant under aseptic conditions in laminar air flow. These sterilized leaves were cut into 2 to 3 mm apical meristem. This apical meristem was cultured aseptically into the bottles of the media. The lid of one of the bottles was removed and the mouth was flamed to avoid further chances of contamination. Explant slice was placed in the bottle with long forceps without touching the rim of the bottle; two to four sections of the explants were placed in each bottle carefully, then it was flamed lightly and tightly sealed. Finally, the name of the sugarcane variety was labeled on the bottle along with the date. All the operations were done under anemic conditions in a laminar air flow cabinet and the weight of the explants was noted.

Incubation of explants

The explants were aseptically cultured on modified MS medium with three auxins supplemented with 2, 4-dichloro phenoxy acetic acid (2, 4-D), naphthalene- acetic acid (NAA) and 4- amino-3,5,6-trichloro-picolinic acid (Picloram) for callus induction at 0.0, 0.5, 1, 2, 3 mgL^{-1}. All the cultures were incubated at 25 ± 2°C and kept under 16 h photo period of florescent tube light in the dark for 4 weeks.

Observations

Each bottle was examined to determine the callus formed from explants. The resulting calli were transferred to fresh medium for further callus proliferation. The callus materials were sub- cultured on the same medium of 2,4-D, NAA and picloram to induce callus for another 4 weeks. After 8 weeks of culture in the presence of 2,4-D, distinctions between regenerable and non- regenerable callus were examined. Regenerable callus has gross appearance, is compacted, has white to cream color and nodular structure, while non regenerable callus has wet appearance, is translucent and brownish in color. The parameters examined included numbers of explants, proliferation of callus, weight of callus, type of callus-regenerable and non- regenerable.

Data analysis

Data were analysed by analysis of variance (ANOVA) using computer software Statistics version 8.1. Complete randomized design (CRD) was used with three treatments and five different concentrations in two factorial designs. Means of callus induction including weight of explants, callus proliferation, callus weight and type of callus were compared; they were statistically significant at α = 5% probability level.

Figure 1.Explant cut into 2-3 mm cultured aseptically into the bottles of the media.

Table 1. ANOVA for callus induction of sugarcane plantlets modulated by different concentration of different auxin.

Source of variation	DF	Mean square		
		Weight of explants	Weight of callus proliferation	Weight of callus.
Varieties	2	0.46203**	1.10021**	2.29702 **
Treatment	2	0.01404 **	1.92311**	4.50119 **
Concentrations	4	0.03061**	0.68875 **	1.69259 **
V x T	4	0.02064**	0.01808 **	0.04923 **
V x C	8	0.04250**	0.02642**	0.05650 **
T x C	8	0.01637**	0.02315**	0.09987**
V x T x C	16	0.01266**	0.00267 ns	0.01152 ns
Error	88			
Total	134	CV. 3.99	CV. 3.95	CV. 4.93

In each column, means followed by common letter are not significantly different at 5% probability level. V= Variety, ns = non- significant, T= treatment, C = concentration, V= co-efficient of variance.

RESULTS AND DISCUSION

Analysis of variance showed that the different levels of auxins had highly significant effects on callus induction. The main parameters used for callus induction are weight of explants, weight of callus, proliferation of callus, as they have direct effect on final callus. The results of ANOVA for callus induction are presented in Table1. Highly significant variations were observed for all parameters of callus induction except variety x treatment x concentration which was non-significant for weight of callus proliferation and weigh of callus ($p < 0.05$).

Weight of explants

Three varieties of sugarcane were used in this experiment. Different varieties have dissimilar weight of disc of explants which also affected the callus formation and regeneration of plantlets. NIA -2012 variety has more weight compared to Gulabi- 95.

Callus proliferation

Callus proliferation was highly influenced by varying

Table 2. Effect of different concentration of 2,4-D, Picloram and NAA on weight of callus proliferation in three sugarcane varieties.

Growth regulators	Concentration (g l^{-1})	Varieties			Mean
		NIA-2012	NIA-105	Gulabi-95	
2,4-D	0.0	0.75^{h-k}	0.70^{l-p}	0.64^{s-v}	0.70^{g}
	0.5	0.88^{c}	0.69^{n-r}	0.63^{t-w}	0.73f
	1.0	0.84^{c-f}	0.71^{k-o}	0.66^{p-t}	0.73^{ef}
	2.0	0.86^{cd}	0.75^{h-l}	0.68^{n-s}	0.76^{b-e}
	3.0	0.95^{b}	0.79^{f-h}	0.72^{j-o}	0.82^{a}
Picloram	0.0	0.70^{m-q}	0.65^{r-u}	0.60^{v-x}	0.65^{h}
	0.5	0.83^{d-f}	0.79^{f-h}	0.73^{j-n}	0.78^{bc}
	1.0	0.78^{g-i}	0.74^{i-m}	0.70^{m-q}	0.74^{d-f}
	2.0	0.80^{e-g}	0.75^{h-l}	0.67^{o-t}	0.74^{d-f}
	3.0	0.87^{cd}	0.71^{k-o}	0.65^{q-u}	0.74^{d-f}
NAA	0.0	0.81^{e-g}	1.09^{a}	0.56^{x}	0.82^{a}
	0.5	0.85^{c-e}	0.78^{g-i}	0.67^{o-t}	0.76^{b-d}
	1.0	0.88^{c}	0.76^{g-j}	0.63^{t-w}	0.76^{c-f}
	2.0	0.97^{b}	0.78^{g-i}	0.61^{u-x}	0.79^{b}
	3.0	0.99^{b}	0.77^{g-j}	0.59^{w-x}	0.78^{bc}
Mean		0.85^{a}	0.76^{b}	0.65^{c}	

In each column, means followed by common letter are not significantly different at 5% probability level. Varieties SE 0.0063), LSD 5%) 0.0127), Concentrations SE 0.0142), LSD 5%) 0.0283), V x C SE 0.0247), LSD 5%) 0.0490).

levels of auxins. Apical meristem was used as explants. Three different genotypes of sugarcane, NIA-2012, NIA-105 and Gulabi-95, were cultured on different MS modified media with three auxins: 2,4-dichloro phenoxy acetic acid (2,4-D), 4- amino-3,5,6-trichloro-picolinic acid (picloram) and naphthalene- acetic acid (NAA). For proliferation of callus, significant variation ($p < 0.05$) was detected for all genotypes (Table 2, Figure 2). Highest weight of proliferation was observed in NIA-2012 (1.41 g) followed by NIA-105 (1.29 g); lowest was in Gulabi-95(1.10 g). The maximum proliferation for 2,4-D was observed in NIA-105 (1.79 g), and minimum in Gulabi- 95 (1.44 g). For picloram maximum weight of proliferation was recorded in NIA-2012 (1.77 g) while minimum was observed in Gulabi-95 (1.3 g). For NAA maximum weight of proliferation was noted in NIA-2012 (1.61 g) and minimum in Gulbi-95 (1.26 g). The highest proliferation of callus was recorded at 3.0 mgL^{-1} for the entire growth regulator hormone used. An efficient and regenerable callus was formed by increasing concentration of auxins. Review of related study by other workers also supports the present results (Table 2) that weight of callus proliferation was enhanced with increase in dose of all the auxins applied. All the concentration gave best results in same combination for the weight of callus proliferation. The results are same with the finding of Khan et al. (2009), Sani (2010); Goel et al. (2010), and Abu et al. (2014). This work is quite different from that of Kenia et al. (2006) who obtained highest proliferation in low concentration of these growth hormones. (Figure. 2 A, B and C labeled on bottle was showing different hormones)

Callus induction

In callus induction different combinations of auxins were used. To obtain the highest role of NAA in callus induction of sugarcane new concentration of more than 1 mgL^{-1} was applied. In sugarcane significant variation ($p < 0.05$) in proliferation of callus was detected for all genotypes (Table 3, Figures 3, 4, 5) different varieties showed variation in callus induction and weight of calli. Highest weight of callus was observed in NIA-2012 (1.41 g) followed by NIA-105 (1.29 g) and lowest in Gulabi-95 (1.10 g). The maximum callus induction weight for 2, 4-D was observed in NIA-105 (1.79 g), and minimum in Gulabi- 95 (1.44 g). For picloram maximum weight of proliferation was recorded in NIA-2012 (1.77 g) while minimum was observed in Gulabi-95 (1.3 g). In case of NAA weight of proliferation was noted for NIA-2012 (1.61 g) and minimum in Gulbi-95 (1.26 g). The highest proliferation of callus was recorded at 3.0 mgL^{-1} for the entire growth regulator hormone used.

Naphthalene acetic acid (NAA) of 2.0 and 3.0 mg/l

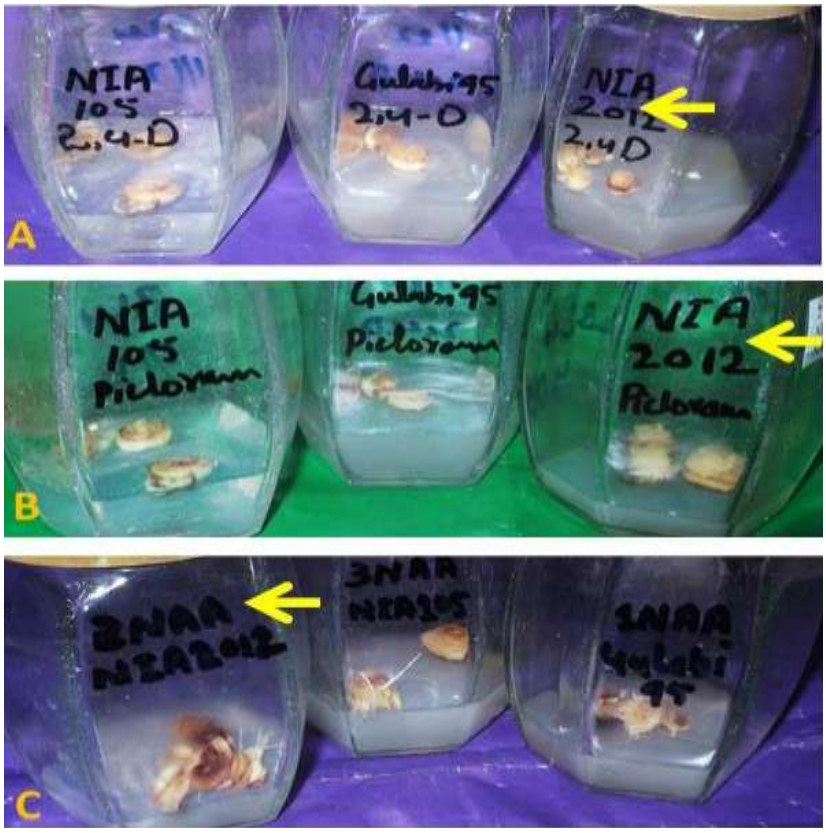


Figure 2.Callus proliferation in NIA-2012, NIA-105 and Gulabi-95 different concentrations of auxins (A= 2,4-D, B= Picloram, C=NAA).

Table 3. Effect of different concentration of 2,4-D,Picloram and NAA on callus induction in three sugarcane varieties.

Growth regulators	Concentration (g l^{-1})	Varieties			Mean
		NIA-2012	NIA-105	Gulabi-95	
2,4-D	0.0	1.01^{q}	0.94^{q-s}	0.82^{uv}	0.92^{l}
	0.5	1.44^{fg}	1.41^{g-i}	1.23^{m-o}	1.36^{de}
	1.00	1.58^{de}	1.55^{de}	1.30^{j-m}	1.47^{c}
	2.0	1.69^{bc}	1.67^{c}	1.33^{i-l}	1.56^{b}
	3.0	1.77^{a}	1.79^{a}	1.44^{g}	1.67^{a}
Picloram	0.0	0.92^{r-t}	0.85^{tu}	0.76^{vw}	0.84^{j}
	0.5	1.31^{j-l}	1.14^{p}	1.01^{q}	1.15^{g}
	1.0	1.43^{gh}	1.34^{i-l}	1.12^{p}	1.29^{f}
	2.0	1.58^{de}	1.38^{g-j}	1.20^{n-p}	1.39^{d}
NAA	3.0	1.7^{ab}	1.63^{cd}	1.30^{k-m}	1.56^{b}
	0.0	0.99^{qr}	0.76^{vw}	0.71^{w}	0.82^{j}
	0.5	1.18^{n-p}	0.96^{q-s}	0.88^{s-u}	1.019^{h}
	1.0	1.33^{i-l}	1.15^{op}	0.99^{qr}	1.16^{g}
	2.0	1.52^{ef}	1.35^{h-k}	1.15^{op}	1.34^{ef}
	3.0	1.61^{cd}	1.44^{g}	1.26^{l-n}	1.44^{c}
Mean		1.41^{a}	1.29^{b}	1.10^{c}	

In each column, means followed by common letter are not significantly different at 5% probability level. Varieties SE 0.0106), LSD 5%) 0.0120), Concentrations SE 0.0236), LSD 5%) 0.0469), V x C SE 0.0409), LSD 5%) 0.0813).

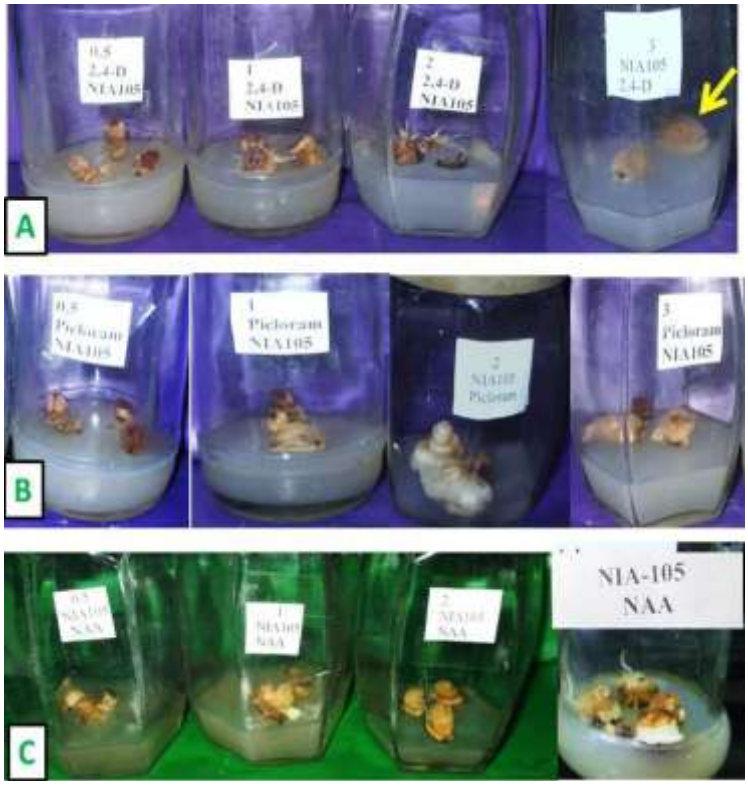


Figure 3. Callus formation in NIA-105 by different concentration of auxins (A= 2,4-D, B= Picloram, C=NAA).

Figure 4. Callus formation in NIA-2012 by different concentration of auxins (A= 2,4-D, B= Picloram, C=NAA).

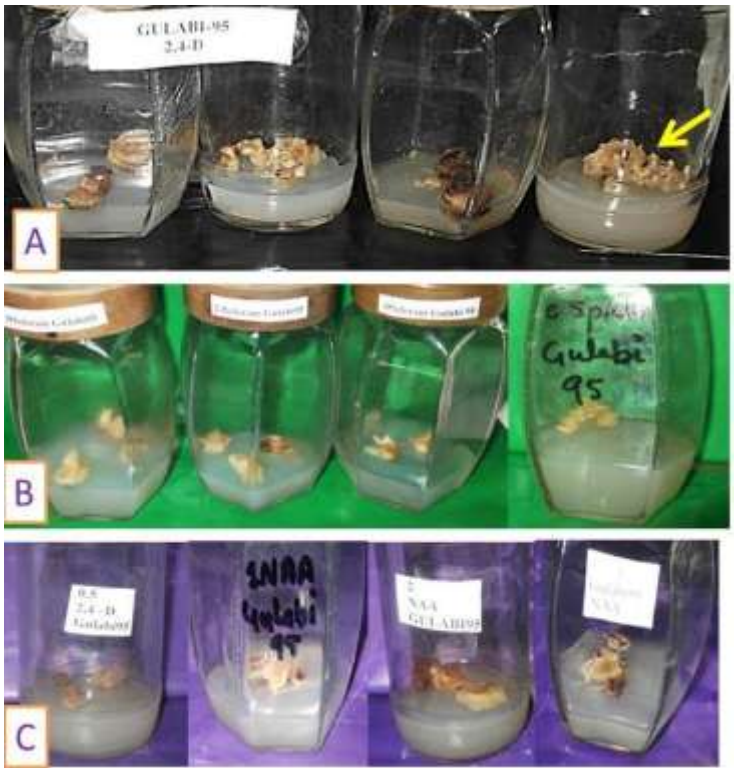


Figure 5. Callus formation in Gulabi-95 by different concentration of auxins (A= 2,4-D, B= Picloram, C=NAA).

produced small amount of non-regenerable and regenerable calli (Figure 6) (Khattak et al. (2014). High concentrations of auxin may be required for higher stages of callus formation. Weight of callus increases with increase in concentration of all the auxins applied, whereas as concentration decreases, weight of callus decreases. All the treatment gave best results at 3.0 mg/l for the weight of callus. Present results are in agreement with the finding of Omarjee et al. (2008), Ather et al. (2009), Khan et al. (2009), Raza et al. (2010), Seema et al. (2011), Khan et al. (2012) and Kazim et al. (2015).

Callus weight and type of callus

Standardized protocol is preferred for callus induction using young maristem as explants of sugarcane varieties. Highly significant variation ($p < 0.05$) in weight of callus was detected for all genotypes (Table 4). The callus induction under the influence of different growth regulators yielded maximum callus in NIA-2012 (2.54 g), followed by NIA-105 (2.34 g) and minimum in Gulabi-95(2.09 g). The maximum callus weight for 2, 4-D was detected in NIA-2012 (3.27 g), and minimum in Gulabi-95(2.67 g). In picloram maximum weight of callus was recorded in NIA-2012 (2.95 g) while minimum was observed in Gulabi-9 (2.3 g). For NAA weight of callus was outstanding for NIA-2012 (2.84 g) and minimum in Gulbi-95 (2.25 g). The highest weight of callus was recorded at 3.0 mgL^{-1} for the entire growth regulator hormone used. Many authors reported best effect of auxins when the callus remained on increased concentration of 2, 4-D applied for prolonged period. Ali et al. (2007) suggested the process of differentiation of regenerable callus and non regenerable callus (Figure.6) based on type of auxin and concentration of auxins. Present work is quite different from that of Gopitha et al. (2010), who found best result of callus induction at lower concentration of NAA, and 2,4-D. Many scientists have used different auxins for callus formation. They found (Table 5) the type of callus depends upon the auxins applied. All the treatment gave best effects at 3.0 mgL^{-1} for the regenerable type of callus. Present results are the same with the finding of Lakshmanan et al. (2006), Valentine et al. (2010), Ijaz et al. (2012), Samiullah et al. (2013), Alcantara et al. (2014). However, this work is different from those of Gandonou et al. (2005), Xing et al. (2010) and Zamir et al. (2012) who obtained regenerable callus at lower concentration of auxins.

Conclusion

This work revealed that the calli obtained from 2, 4-D and picloram produced more genetic variability compared to the calli of NAA. Callus was observed on the basis of

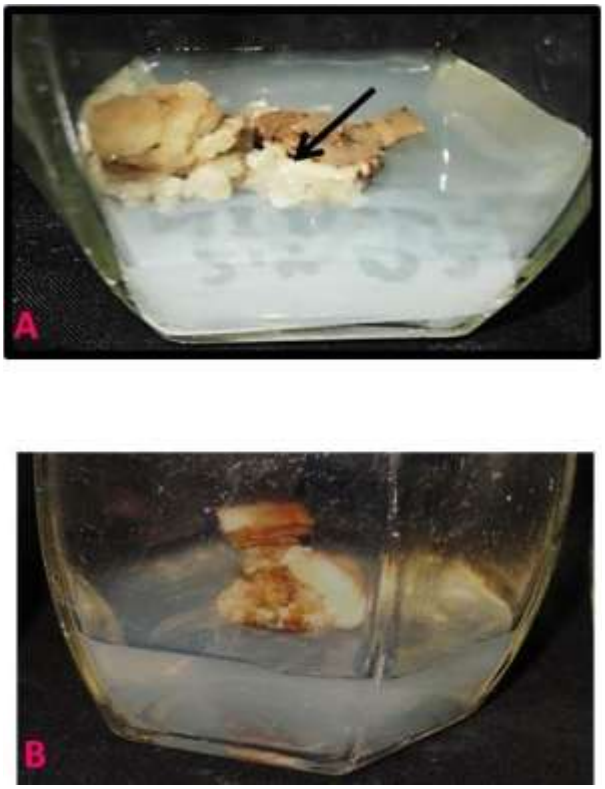


Figure 6. Types of callus obtained by application of different auxins (A= Regenerable. B= Non-regenerable)

Table 4. Effect of different concentration of 2,4-D, Picloram and NAA on callus weight induction in three sugarcane varieties.

Growth regulators	Concentration (g l^{-1})	Varieties			Mean
		NIA-2012	NIA-105	Gulabi-95	
2,4-D	0.0	2.23^{k-o}	2.06^{o-s}	1.92^{r-t}	2.07^{hi}
	0.5	2.23^{k-o}	2.00^{p-t}	2.07^{n-r}	2.10^{g-i}
	1.0	2.78^{c-f}	2.63^{f-i}	2.38^{jk}	2.60^{de}
	2.0	2.97^{b}	2.84^{b-e}	2.52^{h-j}	2.77^{b}
	3.0	3.27^{a}	2.96^{bc}	2.67^{e-h}	2.97^{a}
Picloram	0.0	1.82^{tu}	1.72^{uv}	1.58^{vw}	1.70^{j}
	0.5	2.34^{j-l}	2.27^{k-m}	1.88^{s-u}	2.16^{gh}
	1.0	2.75^{d-g}	2.58^{g-i}	2.16^{l-p}	2.49^{e}
	2.0	2.87^{b-d}	2.77^{d-f}	2.27^{k-m}	2.64^{cd}
	3.0	2.97^{b}	2.85^{b-e}	2.39^{jk}	2.74^{bc}
NAA	0.0	1.58^{vw}	1.43^{wx}	1.32^{x}	1.44^{k}
	0.5	2.24^{k-n}	1.96^{q-t}	1.85^{tu}	2.01^{i}
	1.0	2.47^{ij}	2.17^{l-p}	1.95^{q-t}	2.20^{g}
	2.0	2.70^{d-g}	2.28^{k-m}	2.11^{m-q}	2.36^{f}
	3.0	2.84^{b-e}	2.60^{f-i}	2.25^{k-n}	2.56^{de}
Mean		2.54^{a}	2.34^{b}	2.09^{c}	

In each column, means followed by common letter are not significantly different at 5% probability level. Varieties SE 0.0242), LSD 5%) 0.0481), Concentrations SE 0.541), LSD 5%) 0.1074), V x C SE 0.0936), LSD 5%) 0.1861).

Table 5. Effect of different concentration of 2,4-D,Picloram and NAA type of callus induction in three Sugarcane varieties.

Growth regulators	Concentration (g l^{-1})	Varieties		
		NIA-2012	**NIA-105**	**Gulabi-95**
2,4-D	0.0	Non –regenerable	Non- regenerable	Non- regenrable
	0.5	Regenrable	Regenrable	Non – regenrable
	1.0	Regenrable glossed, white	Regenrable glossed,	Regenrable cream color
	2.0	Regenrable compact,	Regenrable nodular	Regenrable, white
	3.0	Regenrable compact, nodular cream color	Regenrable glossed aspect, white	Regenrable compact, nodular
Picloram	0.0	Non- regenrable	Non – regenrable	Non – regenrable
	0.5	Regenrable	Non – regenrable	Non – regenrable brown
	1.0	Regenrable compact, nodular	Regenrable compact	Regenrable,nodular
	2.0	Regenrable	cream color	, white
	3.0	Regenrable compact, white	Regenrable compact, nodular	Regenrable , glossed, white
NAA	0.0	Non – regenrable	Non- regenrable	Non – regenrable
	0.5	Non – regenrable	Non - regenrable translucent	Regenrable, cream color
	1.0	Regenrable	Regenrable	Regenrable, nodular
	2.0	Non – regenrable	Regenrable nodular	Non - regenrable translucent
	3.0	Regenrable nodular	Regenrable white	Regenrable white

external appearance (regenrable, non- regenrable). The capacity to produce regenerable callus depends on growth hormone.

Conflict of interests

The authors have not declared any conflict of interests.

REFERENCES

Alam R, Mannan SA, Karim Z, Amin MN (2003). Regeneration of sugarcane (*Sacchrum officinarum* L.) plantlet from callus. Pak. Sugar J. 18:15-19.

Alcantara GBD, Dibax R, Filho JCB, Dars E (2014). Plant regeneration and histological study of the somatic embryogenesis of sugarcane (Saccharum spp.) cultivars RB855156 and RB72454. 36:1.

Ali S, Iqbal J, Khan MS (2010). Genotype independent *in vitro* regeneration system in elite varieties of sugarcane. Pak. J. Bot. 42(6):3783-3790.

Ather A, Khan S, Rehman A, Nazir M (2009). Optimization of the protocols for the callus induction, regeneration and acclimatization.

Behara KK, Sahoo S (2009). Rapid *in vitro* Micropropogation of sugarcane(Saccharum officinarum L.. L.CV-Narayana) through callus culture. Nat. Sci. 7:146-147.

Brumbley SM, Snyman SJ, Gnanasambandam A, Joyce P, Hermann SR, da Silva JAG, McQualter RB, Wang ML, Egan BT, Patterson AH, Albert HH, Moore PH (2008). Sugarcane. In: Kole, C. and Hall, T.C. (eds.) A Compendium of Transgenic Crop Plants: Sugar, Tuber and Fiber Crops 7, Blackwell Publishing, Oxford. pp. 1-58.

Cha-um S, Hien NT, Kirdmanee C (2006). Disease free production of sugarcane varieties (*Succharum officinarum* L.) using in vitromeri-stem culture. Asian Network Sci. Info. Pub, Biotechnol. 5(4):443-448.

Coax M, Hogarth M, Smith G (2000). Cane breeding and improvement. In: Manual of cane growing, Hogarth, M. and P. Allsopp (eds). Bureau of Sugar Experimental Stations, Indooroopilly, Aust. pp. 91-108.

Czarinikow G (2010). A First Look at the 10/11 Balance Sheet: A Return to Surplus?. Global sugar production expected to reach record.

Fitch MMM, Lehre AT, Komor E, Moore PH (2001). Elimination of sugarcane yellow leaf virus from infected sugarcane plants by meristem tip culture visualized by tissue blot immunoassay. Plant Pathol. 50:676-680.

Gandonou CB, Bada F, Gnancadja L, Abrini J, Skali-Senhaji N (2005). Effects of NaCl on Na+, Cl- and K+ ions accumula- tion in two sugarcane (*Sacchrum officinarum* L.) cultivars differing in their salt tolerance. Int. J. Plant Physiol. Biochem. 3:55-162

Geijskes, RJ, Wang L, Lakshmanan P, McKeon MG, Berding N, Swain RS, Elliott AR, Grof CPL, Jackson JA, Smith GR (2003). Smartsett Seedlings: Tissue cultured seed plants for the Australian sugar industry. Sugarcane Int. May/June 2003: 13-17.

Goel Y, Singh VP, Lal M, Shairma ML (2010). *In vitro* 9 explants9esis in leaf sheath explants of sugarcane 9xplan var. cos 99259, sugarcane. Technol, 12 (2): 172-175.

Gopitha K, Bhavani AL, Senthilmanickam J (2010). Effect of the different auxins and cytokinins in callus induction, shoot, root regeneration in sugarcane. Int. J. Pharma Bio Sci. 1(3):975-6299.

Ijaz s, Rana IA, Khan IA, Saleem M (2012). Establishment of an in vitrc regeneration system for genetic transformation of selected sugarcane genotypes. Gene. Mol. Res. 11(1):512-530.

Jahangir GZ, Nasir IA, Sial RA, Javid MA, Husnain T (2010). Various Hormonal Supplementations Activate Sugarcane regeneration *In-Vitro*. J. Agric. Sci. 2:4.

Kalunke RM, Kolge AM, Harinathbabu K, Prasad DT (2009). Agrobacterium mediated transformation of sugarcane for borer resistance using Cry 1Aa3 gene and one step regeneration of transgenic plants. Sugar Tech, 11(4): 355-359.

Kazim A, Ghulam R, Zahid M, Shahid M, Shaheen A (2015). Ideal *in-vitro* culture and selection conditions for sugarcane genetic transformation. Pak. J. Agric. Sci. 52(1):43-49.

Khan IA, Dahot MU, Seema N, Yasmin S, Bibi S, Raza S, and Khatri A, (2009). Variability in sugarcane plantlets developed through *in vtiro* mutagenesis. Pak. J. Bot. pp. 153-166.

Khan IA, Khatri A, NizamaniGS, Siddiqui MA, Khanzada MH, Dahar NA, Seema N, Naqvi MH (2004). *In vitro* studies in sugarcane. Pak. J. Biotechnol. 1:6-10.

Khattak WA, Islam U M, Ihsan U (2014). Effect of different media concentrations on colagensis in sugarcane. Afr. J. Biotechnol. 13(11): 1219-1222.

Lakshmanan P, Geijskes RJ, Wang LF, Elliote A, Golf CPL, Berding N, Smith GR (2006). Developmental and hormonal regulation of direct shoot organogenesis and somatic embryogenesis in sugarcane (Saccharum spp. interspecific hybrids) leaf culture. Plant Cell Rep. 25(10):1007-1015.

Mackintosh D (2000). Sugar milling. In: Manual of cane growing. Hogarth M, Allsopp P (Eds.). Bureasu of sugar experimental stations. Indooroopilly, Aust., pp. 369-377

Naz S (2003). Micropropagation of promising varieties of sugarcane and their acclimatization response. Activities on Sugar Crops in Pakistan. In: Proc. Fourth Workshop Res. Dev. pp. 1-9.

Nieves F, Sagarra R, Gonzalez Y, LezcanoM, Cid M, Blanco A, Castillo R (2008). Effect of exogenous arginine on sugarcane (Saccharumsp.) somatic embryogenesis, free polyamines and the contents of the soluble proteins and proline. Plant Cell Tissue Organ Cult. 95:313-20.

Omarjee J, Rutherford RS, Watt DA (2008). South African Sugarcane Research Institute: embracing biotechnology for crop improvement. Sugar Tech 10(1):1-13

Pandey RN, Singh SP, Rastogi J, Sharma ML, Singh RK (2012). Early assessment of genetic fidelity in sugarcane (*Saccharum officinarum*) plantlets regenerated through direct organogenesis with RAPD and SSR markers. Aust. J. Crop Sci. 6(4):618-624.

Parmessur Y, Aljanabi S, Saumtally S, Dookun-Saumtally A (2002). Sugarcane yellow leaf virus and sugarcane yellows phytoplasma: elimination by tissue culture. Plant Pathol. 51:561-566.

Raza G, Ali K, Mukhtar Z, Mansoor S, Arshad M, Asad S (2010). The response of sugarcane (*Saccharum officinarum* L.) genotypes to callus induction, regeneration and different concentrations of the selective agent (Geneticin -418). Afr. J. Biotechnol. 9:8739-8747.

Raza S, Qamarunnisa S, Jamil I, Naqvi B, Azhar A and Qureshi JA (2014). Screening of sugarcane somaclones of variety BL4 for agronomic characteristics. Pakistan Journal Botany. 46(4): 1531-1535

Samiullah (2013). Expansion Of Built Up Area And Its Impact On Urban Agriculture: A Case Study Of Peshawar Pakistan. PhD Thesis, University of Peshawar,

Seema G, Pande HP, Lal J, Madan VK (2011).Plantlet regeneration of sugarcane varieties and transient GUS expression in calli by electroporation. Sugar Technol. 3(1-2):27-33.

Sengar K (2011). Developing an efficient protocol through tissues culture technique for sugarcane micro propagation. Bio Infobank 18:56.

Shahid MR, Khan FA, Saeed A, Aslam M, Rasul F (2012). Development of somaclones in sugarcane genotype BF-162 and assessment of variability by random amplified polymorphic DNA (RAPD) and simple sequence repeats (SSR) markers in selected red rot resistant somaclones. Afr. J. Biotecnol. 11(15):3502-3513.

Sharma M (2005). *In Vitro* Regeneration Studies of Sugarcane. M.Sc. Dissertation Submitted To Thapar Institute of Engineering and Technol, Patiala, India.

Singh B, Yadav GC, Lal M (2003). An efficient protocol for micropropagation of sugarcane using shoot tip explants. Sugar Tech 3(3):113-116.

Smiullah, Khan FA, Abdullah, Ifithkhar R, Raza MM, AslamR,Hammad G, Ijaz A, Maqsood RH and Ijaz U (2013). Callogenesis and Organogenesis studies in some accessions of *Saccharum officinarum L. L.* J. Agric. Sci. Vol. 5, E-ISSN 1916-9760.

Tai PYP, Miller JD (2001). A core collection for *Saccharum spontanium* L. from the world collection of sugarcane. Crop Sci. Soc. Am. 41:879-885.

Thom M, Maretzki A, Komor E, Sakai WS (1981). Nutrient uptake and accumulation by sugarcane cell cultures in relation to the growth cycle. Plant Cell Tissue Organ Cult. 1:3-14.

Wongkaew P, Fletcher J (2004). Sugarcane white leaf phytoplasma in tissue culture: long- term maintenance, transmission, and oxytetracycline remission. Plant Cell Rep. 23:426-434.

Xing ZY, Yuan Y, Wang YH, Zheng LP (2010) .Regenerating Plants from in vitro culture of Erigeron Breviscapus leaves. Afr. J. Biotechnol. 9(26):4022-4024.

Zamir R, Khalil SA, Shah ST, Khan MS, Ahmed K, Shahenshah, and Ahmed N (2012). Efficient *in vitro* regeneration of sugarcane from bud Explants. Biotech Biotech EQ.3094-3099.

academicJournals

Vol. 15(29), pp. 1551-1558, 20 July, 2016
DOI: 10.5897/AJB2016.15455
Article Number: A64368559631
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Optimizing the size of root cutting in *Melia volkensii* Gürke for improving clonal propagation and production of quality planting stock

So Hanaoka[1]*, Mineko Ohira[2], Michinari Matsushita[2] and Jason Kariuki[3]

[1]Hokkaido Regional Breeding Office, Forest Tree Breeding Center, Forestry and Forest Products Research Institute, 561 Bunkyoudaimidorimachi, Ebetsu City, Hokkaido, 069-0836, Japan.
[2]Forest Tree Breeding Center, Forestry and Forest Products Research Institute, 3809-1 Ishi, Hitachi City, Ibaraki, 319-1301, Japan.
[3]Kenya Forestry Research Institute, P.O. Box 20412 – 00200, Nairobi.

Received 6 May, 2016, Accepted 11 July, 2016.

Melia volkensii **is a tree species growing in semi-arid regions of East Africa, and is recognized as one of the most valuable forestry species in such regions of Kenya. The use of root cutting is an easy method for its clonal propagation, but the most appropriate conditions have not been systematically examined. In this study, the relationship between diameter of root cut edge or fresh weight and formation frequency of adventitious bud were assessed. In addition, roots were divided into two fragments, and formation frequency of adventitious bud was compared between cross-sections roots without root tip and roots with root tip. Both the diameter of the cut edge and fresh weight of roots forming adventitious buds were significantly higher than in those not forming them (unpaired *t*-test, p<0.01). Formation frequency of adventitious bud was 77.0% in roots satisfying the criteria of cut edges diameter >15 mm and fresh weight >20 g, but it decreased (37.2%) in roots that did not meet this criteria. Based on anatomical analysis, an effect of the developmental stage of root fragments on adventitious bud formation was suggested, and root size is thought to reflect the stage of root development. The formation frequency of adventitious buds was 72% even in cross-sectioned roots, and the proportion was not significantly different from that of normal root tip (p>0.05). Therefore, it is concluded that root cutting using cross-sectioned roots are also available for clonal propagation.**

Key words: Clonal forestry, anatomical analysis of root, availability of cross-sectioned roots.

INTRODUCTION

Melia volkensii Gürke is a deciduous, broad-leaved monoecious tree species distributed in semi-arid regions

*Corresponding author. Email: sohana@affrc.go.jp. Tel: +81-11-386-5087. Fax: +81-11-386-5420.

of east Africa (Orwa et al., 2009). The tree is recognized as one of the most valuable species for agroforestry purposes in the area because of features such as its drought tolerance, fast growth, high wood quality and ant resistance (Kariuki et al., 1990; Mulanda et al., 2015). Plus trees have been selected in Kenya, and improvement for drought tolerance and growth rate are being attempted through a breeding program (Muchiri and Mulatya, 2004; Luvanda et al., 2015). Superior varieties will be developed in the near futures, and the availability of clonal propagation protocol has important implications in the distribution of these varieties. This is because cloned trees maintain the genetic makeup of their mother trees, and the use of clones with homogeneous and desirable traits will improve productivity in forestry and agroforestry (Park et al., 1998; McKey et al., 2009). It is known that succession rate of stem cutting is not necessarily high (up to 33%) in *M. volkensii* although the reason is unclear (Indieka and Odee, 2004). Grafting is generally used as the clonal propagation method in *M. volkensii*. However, the method requires an investment in time and effort to prepare the plant materials, and a practiced technique and dedicated tools are also required. Therefore, other easy and cost-effective method for clonal propagation is needed for *M. volkensii*.

Root cutting is one of the general clonal propagation method, and it's use have been reported in various species such as *Populus tremuloides* (Schier, 1974; Snedden et al., 2010), *Populus tremula×Populus tremuloides* (Stenvall et al., 2004), *Paulownia tomentosa*, *Paulownia fortunei* (Ede et al., 1997), *Prunus avium* (Ghani and Cahalan, 1991) and *Detarium microcarpum* (Ky-Dembele et al., 2010). These studies have identified the effects of some factors on the succession of root cutting. For example, Ede et al. (1997) reported the importance of root size (whether > 5 mm of diameter of root cut edge or not) and the position where the root was obtained. Ky-Dembele et al. (2010) also reported significant effect of root size (> 10 cm of length and 15 mm of diameter) and no effect of root alignment on succession of root cutting. These studies indicate that optimum condition for root cutting should be considered in accordance with root configuration or other attributes of the target species.

A high ability to produce root suckers in *M. volkensii* is associated with mechanical damage to the root (Stewart and Blomley, 1994), and viability of root cutting was also reported by Mulatya et al. (2002). In addition, *M. volkensii* develop thick, deep roots (Mulatya et al., 2002) and therefore, clonal propagation using the abundant root resources is feasible. However, essential conditions for successful root cutting have not been systematically tested in *M. volkensii*, but very important knowledge for efficient clonal propagation. The present study evaluated: 1) the relationship between root size and formation of adventitious buds, 2) the anatomical differences between roots on which adventitious buds formed or did not form, to understand the anatomical factor needed for successful root cutting, and 3) the usefulness of cross-sectioned roots without root tips for enhancing the clonal propagation efficiency of *M. volkensii*.

MATERIALS AND METHODS

Experiment 1: Effect of root size on efficiency of adventitious bud formation

The relationship between root size (diameter of cut edge and fresh weight) on the efficiency of adventitious bud production was examined. Plant materials were potted one-year-old juvenile trees of *M. volkensii*, derived from open-pollinated seeds collected in Kenya. The plants were grown in pots in temperature-controlled greenhouse with natural light in Hitachi, Japan (36.69N, 140.69E). One to four root tips of about 8 to 16 cm length were cut from each of the 48 juvenile trees with tree height of about 40 cm (Figure 1a). The diameter of the cut edge (average of two sides) and fresh weight were measured by caliper and electronic balance, respectively. Root length was not measured in this study because it was difficult to measure curved root fragments (Figure 1a). In total, 94 roots were randomly selected, and these roots were planted in polypots (diameter and height are 10 and 21 cm, respectively) with mixed soil (Yabashi et al., 1992; Kanuma soil: leaf mold = 1:2 volume ratio). Note that, about 0 to 5 mm of proximal end was exposed above ground (Figure 1b). They were kept in the above mentioned greenhouse, watered every two to three days until the 60th day after planting, and then the presence of adventitious buds was observed. Temperature and humidity in greenhouse during the experiment period were 24.9±3.5°C (mean±SD) and 75.2±17%, respectively. The difference in root size for roots with and without adventitious buds was tested by unpaired *t*-test using the *t test* function in R software (R Development Core Team 2015).

Experiment 2: Attempt at promotion of propagation

The suitability of cross-sectioned roots for improving the efficiency of propagation was tested. Roots were cut from four *M. volkensii* trees with diameter at breast height of about 3 cm that had been grown in the greenhouse (potted 3-year-old trees with height controlled at about 2 m). The roots were subjected to two kinds of treatments, that is, cross-section of roots without root tips (hereafter called C-S roots) and roots with root tips (hereafter called RT roots) were used for the experiments. The length of both kinds of roots was about 5 to 10 cm. In total, 22 C-S and 17 RT roots were used for the experiment. Environmental condition and subsequent procedures until observation of adventitious buds were the same as experiment 1. When C-S roots were planted, distal end was always buried in soil. If proximal end was buried in soil, adventitious bud would be formed in the soil as was found in the preliminary studies (Figure 2).

Testing for the difference in the proportion of roots forming adventitious bud in C-S and RT roots was carried out by *prop.test* function in R software (R Development Core Team, 2015).

Anatomical observation of adventitious bud formation

To reveal difference between adventitious buds formed and not formed roots, anatomical observation was carried out by frozen

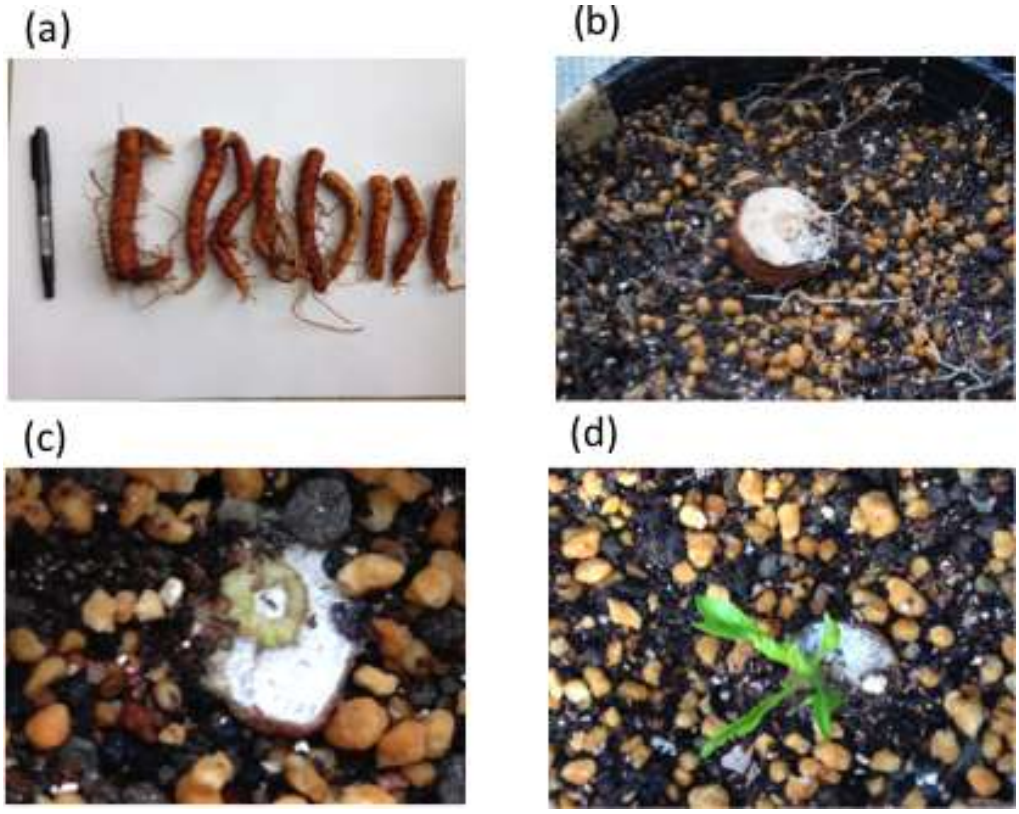


Figure 1. Representative root fragments used for experiment 1 (a), planted root fragments; (b), callus on root cut edge; (c) adventitious buds on callus (d).

section technique (Kawamoto, 2003). Three samples were selected from roots both freshly forming and not forming adventitious buds. The roots were placed in a stainless steel container filled with SCEM-L1 embedding gel (Section-lab Co. Ltd., Japan) and frozen in cooled n-hexane by liquid nitrogen. The frozen blocks were removed from the container, and sectioned at a thickness of 10 µm using a CM3050S cryomicrotome (Leica Microsystems, Germany). The sections were stained with 0.5% toluidine blue or iodine-potassium iodide solution. The stained sections were observed under a DMRXE light microscope (Leica Microsystems, Germany) and photographed by a digital camera. The diameters of cut edges and secondary xylems were measured by a digital caliper. Regarding projected area of starch in secondary xylem or secondary phloem and cortex, two to five photos (size: 2048 × 1536 µm) of the sections stained with iodine-potassium iodide solution were randomly taken in each sample, and the photos converted to binary images to cover areas of starch grains using the software ImageJ 1.48v. The rates of starch (black) area were also calculated by the measure command of ImageJ software.

RESULTS

Effect of root size on efficiency of adventitious bud formation

Green, nodular and compact callus formation was observed within two to four weeks on most roots eventually forming adventitious buds (Figure 1c); adventitious buds developed from the callus (Figure 1d). The relationship among diameter of root cut edge, fresh weight and existence of adventitious buds is shown in Figure 3. Adventitious buds were observed in 50 out of 94 roots (53.2%).

Both the diameter of the cut edge and fresh weight were significantly different between roots that formed adventitious buds and those that did not form them ($p < 0.01$), and roots that formed adventitious buds were larger than those that did not form them (Figure 4).

Effect of sectioning of root cutting on adventitious bud formation

Adventitious buds were observed in 16 out of 22 (72.7%) C-S roots and 16 out of 17 (94.1%) RT roots. The relationship between the diameter of root cut edge, fresh weight and existence of adventitious buds is shown in Figure 5. The proportion of roots forming adventitious buds was not significantly different between C-S and RT roots ($p > 0.05$).

Figure 2. An example of root with adventitious bud produced in soil and grown.

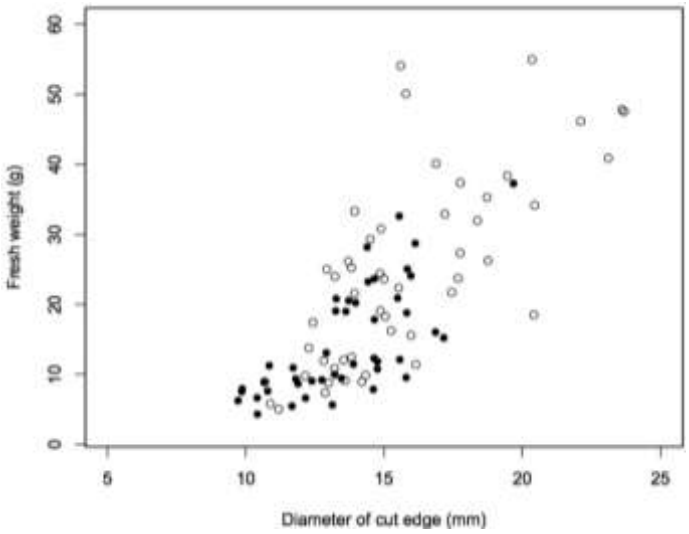


Figure 3. Relationships between diameter of root cut edge, fresh weight and adventitious bud formation in experiment 1. Open and filled circles indicate roots forming and not forming adventitious buds, respectively.

Anatomical observation of adventitious buds

Secondary xylem was observed in the core of roots (Figure 6a). The diameter of secondary xylem was larger in roots forming adventitious buds (4.0, 7.3, and 7.7 mm) than in those that did not form adventitious buds (2.6, 2.8 and 3.5 mm) (Table 1). Secondary xylem and vascular cambium developed in all cut roots forming adventitious buds (Figure 6b). On the other hand, interfascicular cambium and vascular cambium were not detected in roots that did not form adventitious buds (Figure 6c). Starch grains were more abundant in the cortex, secondary phloem (Figure 6d) and secondary xylem (Figure 6f) of roots that formed adventitious buds (Figure 6d, f and Table 1). Most vessels were occluded in roots that did not form buds (comparison between Figure 6h and i).

DISCUSSION

Effect of root size on efficiency of adventitious bud formation

In experiment 1, the frequency of formation of adventitious buds was higher in larger roots; both the diameter of the cut edge and fresh weight of roots forming adventitious buds were significantly higher than in those that did not form them (Figure 4). Therefore, root size (volume) is thought to be important for the success of root cutting in *M. volkensii*. For practical purposes, a cut edge larger than 15 mm and fresh weight greater than 20 g will be criteria to follow because the frequency of formation of adventitious buds was 77.0% under these conditions. On the other hand, it decreased to 37.2% in roots that did not meet these criteria.

Possible factors affecting adventitious bud formation in root cutting of *M. voleksnii*

The effect of root size on the formation frequency of adventitious buds has been reported in some species [for example, *Actinidia chinen*sis (Lawes and Sim 1980), *Prunus avium* (Ghani and Cahalan, 1991), *Detarium microcarpum* (Ky-Dembele et al., 2010)], and the importance of nutritive conditions correspond to root size in some of them. In *M. volkensii*, starch content tended to be high in roots forming adventitious buds (Figure 6d-g and Table 1). However, it varied in roots that did not form adventitious buds. In populus, it is reported that

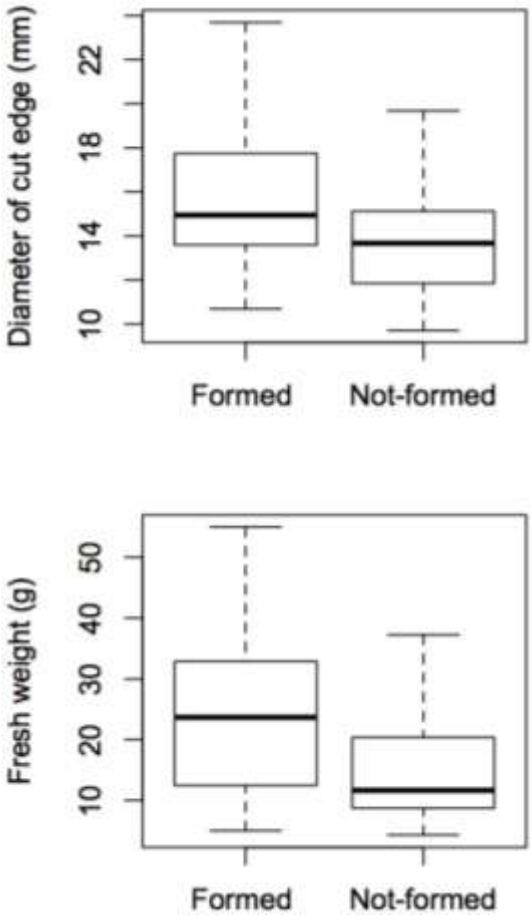


Figure 4. Differences in size of root cut edge (a) and fresh weight (b) between roots forming and not forming adventitious buds.

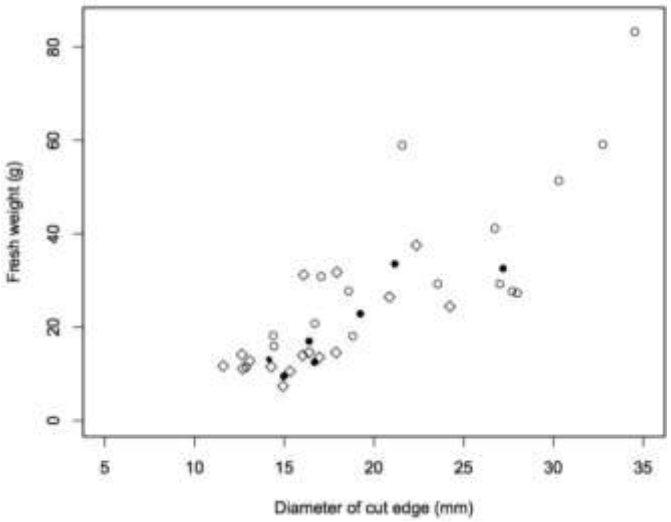


Figure 5. Relationship between diameter of root cut edge, fresh weight and adventitious bud formation in experiment 2. Open and filled circles indicate C-S roots forming and not forming adventitious buds, respectively. Open and filled diamonds indicate RT roots forming and not forming adventitious buds, respectively.

carbohydrate is not necessarily a determining factor for successful root cutting, and the importance of hormones or unknown substances in roots was reported (Snedden et al., 2010). A similar phenomenon is thought to be applicable to *M. volkensii*, and starch accumulation may be a factor, though not a major one, for successful root cutting.

Three samples were selected for anatomical observation of roots both forming and not forming adventitious buds. Callus and adventitious buds usually formed around the cambium between the secondary xylem and cortical layer in *M. volkensii,* and the vascular cambium could be clearly distinguished in roots forming adventitious buds (Figure 6b). This means that mature roots in secondary growth stage might be easy to form adventitious bud. On the other hand, the vascular cambium was indistinct in roots not forming adventitious buds (Figure 6c). The average diameter of the secondary xylem was 2.4 mm in roots that did not form buds (n=3), which was smaller than that of roots that formed adventitious buds (5.7 mm, n=3). Therefore, roots that did not form adventitious buds are thought to be transition stage from primary growth to secondary growth, which might affect the efficiency of adventitious bud formation. This study findings on more bud induction in larger roots indicated that root size might reflect the effect of the developmental stage of the root. In addition, it was observed that the vessels in one of the roots that did not form adventitious buds appeared to be filled by tylose or a similar substance, an indication of infection, water stress and/or senescence (Phillips et al., 1987; Neuhaus et al., 2007). However, only a limited number of samples could be observed in this study, and therefore, the relationship between root size, developmental stage and the resulting efficiency of formation of adventitious buds should be observed concurrently using more abundant samples in future studies.

The effect of some factors other than root size has also been reported in other species. For example, the effect of clonal variation (Stenvall et al., 2004) or temporal variation on efficiency of root cutting has also been reported (Snedden et al., 2010). In this study, many unknown tree families were used in experiment 1, and therefore, clonal variation could not be considered as part of the experiment. On the other hand, the efficiency of adventitious bud formation was confirmed in each family in experiment 2, and all families showed a high rate of formation of adventitious buds (80.0, 66.7, 80.0 and100% for the four mothers). Therefore, clonal variation was not observed, but a high tendency for root suckering in *M. volkensii* was confirmed. There may also be phenological

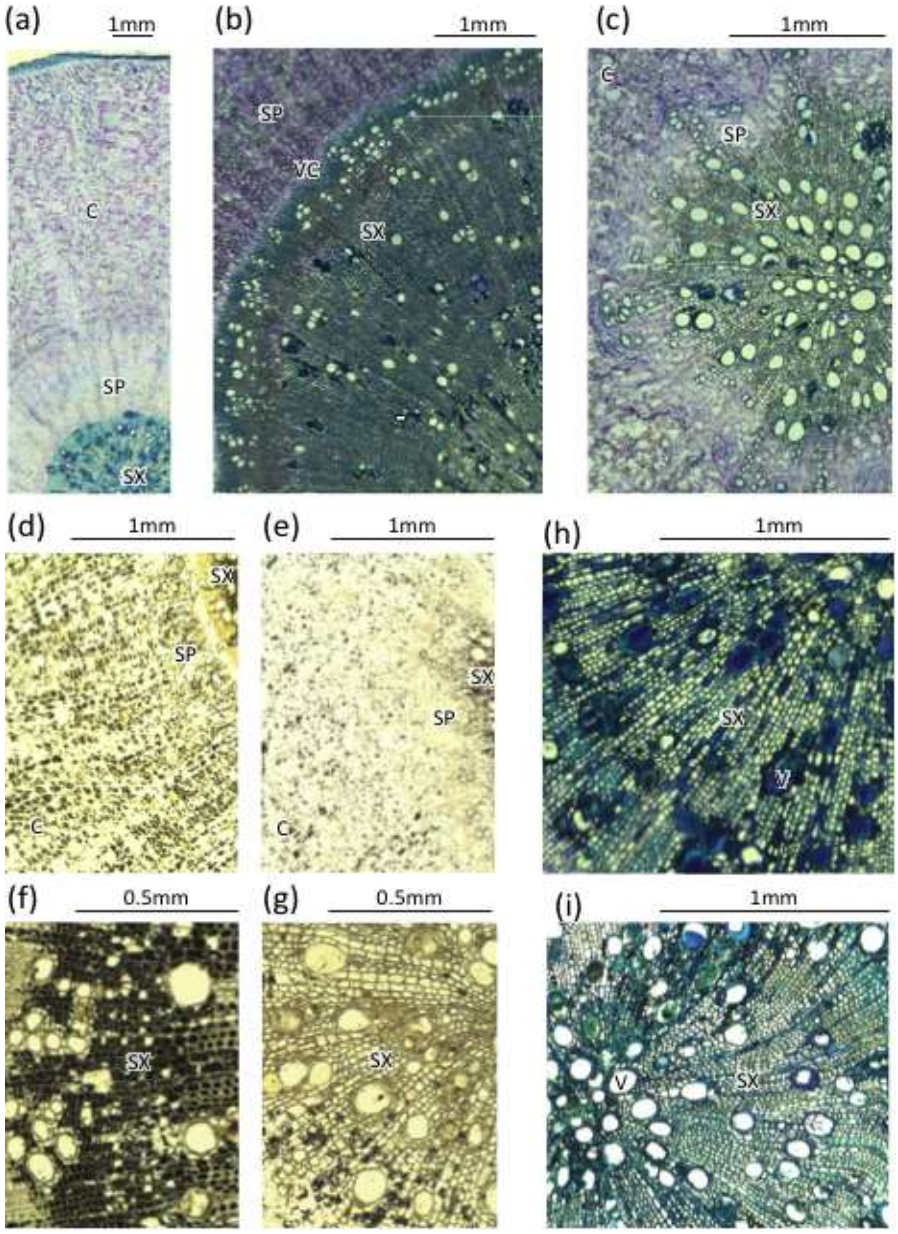


Figure 6. Representative cross-section of roots. (a) Vascular cambium in a root forming adventitious buds; (b), undeveloped vascular cambium from a root not forming adventitious buds; (c), starch (dark granules) distribution in secondary phloem and cortex of a root forming adventitious buds; (d), secondary phloem and cortex of a root not forming adventitious buds (e); secondary xylem of a root forming adventitious buds (f); (g), secondary xylem of a root not forming adventitious bud; (h), vessel embolism in a root not forming adventitious buds; (i), open vessels in a root forming adventitious buds. (C) cortex; (SP) secondary phloem; (SX) secondary xylem; (VC) vascular cambium; (V) vessel are also shown in each figure.

effects: *M. volkensii* grows in east Africa, and clear rainy and dry seasons alternate in the region. Therefore, hormones and metabolic resources in roots will be different in different seasons of root collection. Unfortunately, source trees were grown in a greenhouse with controlled temperatures and watering conditions, and therefore, other conditions could not be tested in this study.

Table 1. Results of anatomical observation

Sample	Formation of *adventitious* bud	Diameter of cut edge (mm)	Diameter of secondary xylem (mm)	Projected area of starch in secondary phloem and cortical layer (%)	Projected area of secondary xylem (%)
1	Yes	22.7	7.7	32.1	30.6
2	Yes	21.2	4.0	37.7	47.0
3	Yes	19.2	7.3	15.7	35.7
4	No	16.5	2.6	27.2	13.9
5	No	14.3	3.5	14.9	5.0
6	No	13.7	2.8	8.5	20.5

Viability of cross-sectioned root for root cutting

In experiment 2, most roots used in this second experiment satisfied the criteria for success root cuttings, that is, >15 mm cut edge and >20 g fresh weight (Figure 5), and formation frequency of adventitious bud were 74.4% in C-S root and 94.1% in RT root. Although the frequency was slightly lower in C-S roots, the values were not significantly different ($p > 0.05$). Therefore, efficient propagation will be achieved by using both C-S and RT roots.

Conclusion

From this study, the importance of root size for successful root cuttings in *M. volkensii* is concluded. Anatomical analysis indicated that root size roughly reflects the developing stage of root, and larger root (> 15 mm of diameter and > 20 g of fresh weight) are recommended for root cutting. Both parameters will be effective in inferring the developing stage of roots because significant correlation between root cut edges and fresh weight was observed (experiment 1, $r^2 = 0.58$, $p < 0.01$). However, diameter of cut edge could be more convenient visual parameter, and therefore, root cut edge will be more convenient parameter for practical utility.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank Ms Kosugiyama and Mr Takakura for their kind support during the experiments. They also thank Mr Takeda, Mr Narumi, Mr Omondi and Dr Muturi for their assistance in writing the paper. This work was carried out as part of a KEFRI/JICA project (Development of Drought Tolerant Trees for Adaptation to Climate Change in Drylands of Kenya).

REFERENCES

Ede FJ, Auger M, Green TGA (1997). Optimizing root cutting success in Paulownia Spp. J. Hort. Sci. Biotechnol. 72:179-186.

Ghani AKMO, Cahalan CM (1991). Propagation of *Prunus avium* from root cutting. Forestry 64:403-409

Indieka SA, Odee DM (2004). Vegetative propagation of *Melia volkensii*: an indigenous multipurpose drylands tree species. In: Kamondo BM, Indieka SA, Odee DW, Muluvi GM, Rao KN, Machuka J (2007). Regeneration of *Melia volkensii* Gürke (Meliaceae) through direct somatic embryogenesis. New Forest. 34:73-81.

Kimondo JM, Mulatya JM, Muturi GM (eds) Recent Mukau (*Melia volkensii* Gürke) Research and Development (Proceedings of the First National Workshop, Kenya Forestry Research Institute (KEFRI), Kitui Regional Research Center), pp. 22-26.

Kariuki JG, Mulaya J, Kimondo J, Ogawa S, Muchiri D (1990). Genetic improvement of *Melia volkensii*: Evaluation of candidate plus trees. http://10.10.20.22:8080//handle/123456789/502

Kawamoto T (2003). Use of a new adhesive film for the preparation of multi-purpose fresh-frozen sections from hard tissues, whole-animals, insects and plants. Arch. Histol. Cytol. 66:123-143.

Ky-Dembele C, Tigabu M, Bayala J, Savadogo P, Boussim IJ, Oden PC (2010). Clonal propagation of *Detarium microcarpum* from root cuttings. Silvae Fennica 44:775-785.

Lawes GS, Sim BL (1980). Kiwifruit propagation from root cuttings. New Zealand J. Exp. Agr. 8:273-275.

Luvanda AM, Musyoki J, Cheboiwo J, Wekesa L, Ozawa M (2015). An assessment of the socio-economic importance of *Melia volkensii* based enterprises in Kenya. Kenya Forestry Research Institute.

McKey D, Elias M, Pujol B, Duputié A (2010). The evolutionary ecology of clonally propagated domesticated plants. New Phytol. 186:318-332

Muchiri D, Mulatya J (2004). Survey of *Melia volkensii* plus trees in the Eastern and Coastal provinces of Kenya. In Kamondo BM, Kimondo JM, Mulatya JM, Muturi GM (eds) Recent Mukau (*Melia volkensii* Gürke) Research and Development (Proceedings of the First National Workshop, Kenya Forestry Research Institute (KEFRI), Kitui Regional Research Center), pp. 10-14.

Mulanda ES, Chuhila Y, Awori RM, Adero MO, Amugune NO, Akunda E, Kinyamario I (2015). Morphological and RAPD-marker characterization of *Melia volkensii* (Gürke) *in vitro* plants regenerated via direct and indirect somatic embryogenesis. Afr. J. Biotechnol. 14:1261-1274.

Mulatya JM, Wilson J, Ong CK, Deans JD, Sprent JI (2002). Root architecture of provenances, seedlings and cuttings of *Melia volkensii*: implications for crop yield in dryland agroforestry. Agrofor. Syst. 56:65-72.

Neuhaus A, Turner DW, Colmer TD, Kuo J, Eastham J (2007). Drying half the root-zoon of potted avocado (*Persea americana* Milll., cv. Hass) trees avoids the symptoms of water deficit that occur under complete root zone drying. J. Hort. Sci. Biotechnol. 82:679-689

Orwa C, Mutua, A, Kindt R, Jamnadass R, Simons A (2009). Agroforestry database: a tree reference and selection guide version

4.0. http://www.worldagroforestry.org/af/treedb/
Park YS, Barrett JD, Bonga JM (1998). Application of somatic embryogenesis in high-value clonal forestry: Deployment, genetic control, and stability of cryopreserved clones. In Vitro Cell. Dev. Biol. Plant 34:231-239.
Phillips D, Grant BR, Weste G (1987). Hisological changes in the roots of an avocado cultivar, Duke 7, infected with *Phytophthora cinnamomi*. Phytopathology 77:691-698
R development core team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. htttp://www.R-project.org
Snedden J, Landhausser SM, Lieffers VJ, Charleson LR (2010). Propagating trembling aspen from root cuttings: impact of storage length and phenological period of root donor plants. New Forest. 39:169-182.
Stenvall N, Haapala T, Pulkkinen P (2004). Effect of genotype, age, treatment of stock plants on propagation of hybrid aspen (*Populus tremula*×*Populus tremuloides*) by root cuttings. Scandinavian J. For. Res. 19: 303-311.
Stewart M, Blomley T (1994). Use of *Melia volkensii* in a semi-arid agroforestry system in Kenya. Commonwealth For. Rev. 73:128-131.
Yabashi S, Amemiya Y, Koh S, Takahashi S, Tanaka Y (1992). Studies on physical properties of horticultural soils I. Characteristics of the pore structure and water retentivity of Kanuma-tsuchi. Tech. Bull. Fac. Hort. Chiba Univ. 46:129-134.

academicJournals

Vol. 15(29), pp. 1559-1565, 20 July, 2016
DOI: 10.5897/AJB2016.15393
Article Number: AB7971559633
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Lipids of Amazon Caimans: A source of fatty acids

Augusto Kluczkovski Junior[1*], Alicia De Francisco[1], Luiz Beirão[1], Ariane Kluczkovski[2] and Heitor Barbosa[3]

[1]Federal University of Santa Catarina, Brazil.
[2]Federal University of Amazonas, Brazil.
[3]Instituto Federal de Amazonas – IFAM, Brasil.

Received 11 April 2016, Accepted 30 June, 2016.

Some species of fish and other aquatic organism are important sources of protein and fatty acids that are beneficial to human health and can be industrially processed. The fatty acid profile of *Caiman crocodilus* and *Melanosuchus niger* (native to the Brazilian Amazon flooded forest) was determined in samples of a commercial cut (tail fillet) and fat (fat body and somatic fat) of these two species. There were no statistically significant differences in the total lipid content between them (p ≥ 0.05) and both had higher levels of palmitic, stearic (saturated), and oleic (unsaturated) acids. However, omega 3 (ω-3) and omega 6 (ω-6) were not detected in the samples of the commercial cut; they were present only in the fats evaluated. Clinical studies are necessary to assess the influence of fatty acids from Amazon Caimans on human diet and the feasibility of obtaining new products such as nutraceuticals.

Key words: Black caiman, spectacled caiman, omega 3, omega 6.

INTRODUCTION

A large number of consumers have had access to food nutritional information. Therefore, there has been a growing interest in some nutrients associated with the prevention of diseases through diet, including fat consumption. Lipids are important constituents of cell membranes and play major role in metabolic processes (Martin et al., 2006). They are composed of fatty acids (FA) of different chain lengths that may be saturated (SFA) or unsaturated (UFA). The unsaturated fatty acids are classified into monounsaturated (MUFA) and polyunsaturated (PUFA) fats (Moreira et al., 2002). The term essential fatty acid (EFA) refers to polyunsaturated fatty acids that must be obtained through foods since they cannot be synthesized in the human body and are required for maintaining good health. Essential fatty acids (EFAs) are divided into two groups: (a) ω-3: which includes the alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and (b) *ω-6*: which includes cis-linoleic acid (LA) (Kaur et al., 2014). Although produced in the human body, oleic acid (ω-9) requires the presence of ω-3 and ω-6. Clinical studies have been performed on EFAs due to their special tendency to be considered as functional foods. According to Siro et al. (2008), functional foods are defined as “food that may provide health benefits beyond basic nutrition”. Despite that, some studies reported the

*Corresponding author. Email: augustokjr@hotmail.com.

western diet as “deficient” in some FA and very low levels of ω-3 PUFAs, leading to an unhealthy ω-6/ω-3 ratio without benefits to human health (Simopoulos, 2008). This ratio is important to be considered in order to prevent diabetes and the risk of cardiovascular diseases (Simopoulos, 2016; Russo, 2009).

As the availability of some EFAs depends on the diet, it is important to know, among the animal species, those that are commercially available. Fish, both saltwater and freshwater, is among the most important sources of FA. However, the amount of EFAs varies between similar species, and according to environmental variables, such as diet and habitat (Ohr, 2005). In addition to fish, the crocodilians black caiman (*Melanosuchus niger*) and spectacled caiman (*Caiman crocodillus*) are important food sources and can be used commercially by native populations (Da Silveira and Thorbjarnarson, 1999).

In the most recent global assessment of International Union for Conservation of Nature and Natural Resources (IUCN), the two species are in the category of least concern (Ross, 2000). These species belong to the family Alligatoridae and are found in South American countries. Like any kind of fauna exploitation, the commercial exploitation of caimans can be performed in a sustainable manner with animals bred in captivity or in their natural habitat. The commercial exploitation of wild crocodilian is well-known in some countries such as Australia (Seafield et al., 2014) and United States of America (Louisiana Department of Wildlife and Fisheries (DWF), 2013). In those programs, the commercial exploitation is a sustainable tool for crocodilian species and their natural habitat. In Brazil, in addition to the Amazon species, the species yacare caiman (*Caiman yacare*) and broad-snouted caiman (*Caiman latirostris*) are also commercially exploited; their meat and by-products have been frequently studied (Canto et al., 2012). Vicente Neto et al. (2007, 2006) carried out studies on *C. yacare* composition and found lipids ranging from 0.4 of 0.54% in the tail. Cernikova et al. (2015) reported lipids ranging from 4.41 to 5.90% in the same commercial cut from Nile crocodile (*Crocodilus niloticus*). Despite the available studies on commercial cuts of other crocodilians, there is no data on the FA profile of the abdominal fat cavity of black caiman and commercial cuts of spectacled caiman.

Accordingly, the aim of this study was to evaluate the FA profile of samples of the spectacled caiman and somatic and fat body of the black caiman, to contribute to scientific data for human nutrition.

MATERIALS AND METHODS

Animal sample and slaughtering procedures

Sixty animals of the species black caiman and spectacled caiman were captured and slaughtered for the purpose of this study, with official authorization granted by the Brazilian Institute for the Environment and Renewable Natural Resources - IBAMA (14498, 1-2/2009). The animals were captured in their natural environment in the Piagaçu-Purus Sustainable Development Reserve, in the municipalities of Anori, Amazonas State, Brazil. They were slaughtered in the high water level season (December) and were handled observing the current legislation and the Humane Methods of Slaughter Act (animal welfare). The samples of fat body were collected during evisceration, manually separated from the mesentery and stored in ice (0°C). After slaughter, the carcasses were submitted to cutting (commercial cuts), as described by Kluczkovski Junior et al. (2015). During the separation of the tail portions, samples of somatic fat were collected in the space between the muscles. All samples were frozen in the fish facility and were sent for analysis. The samples were classified into: (a) muscle tissue (tail fillet of both species) and (b) adipose tissue: somatic and body fat of Black caiman. Huchzermeyer (2003) previously reported the terms fat body and somatic fat.

Fatty acid profile analysis

The samples were minced using an industrial blender until homogeneous mass was obtained, and total lipids were estimated by Soxhlet. The assays were performed in triplicate according to Association of analytical communities (AOAC) (2005). For the analysis of FA, total lipids were extracted according to procedures described by Folch et al. (1957), and the preparation of FA methyl esters were carried out according to Hartman and Lago (1973). Briefly, FA were saponified with a methanolic NaOH solution and methylated under acidic conditions by adding a solution of ammonia chloride, methanol and sulphuric acid. The FA methyl esters were submitted to gas liquid chromatography on a GC-2014 chromatographer (Shimadzu Corporation, Kyoto, Japan), equipped with a flame ionization detector (FID) and a capillary column of 10% cyanopropylphenyl-90% biscyanopropylpolysixolano 105 m, 0.25 mm ID, 0.2 µm df (Restek®) in the following conditions: Injector: 260°C; Detector: 260°C; Column: 140 initial (5 min); 2.5 to 240°C (15 min)/60 min. Individual FA were expressed as percentage of the chemical components expressed on dry matter basis (DM) and the limit of detection (LOD) for the FA was 0.01%.

Statistical analysis

A non-parametric test, the Wilcoxon-Mann-Whitney U test (Bauer, 1972; Hollander and Wolfe, 1999) was used to compare the sample species instead of using the alternative Student T-test, which has to be applied on independent samples. In the FA profile analysis of *black caiman*, the normal distribution of frequencies was verified using the Shapiro-Wilk W test (Razali and Wah, 2011), and the homogeneity of variances was verified using the Fligner-Killeen test (Conover et al., 1981). All descriptive and inferential statistical tests were carried out using the R software (R Core Team, 2015).

RESULTS AND DISCUSSION

The total lipid content of black caiman and spectacled caiman, as well as data of other species of crocodilians reported by other authors, are presented in Table 1. There were no statistically significant differences in the total lipid content between the two species ($p \geq 0.05$). The lipid content in the spectacled caiman (0.02 g%) was lower than that in the black caiman (0.6 g%). This can be explained by considering that the samples were obtained from wild animals whose availability of food varies

Table 1. Fat acid profile in commercial cut (tail) of *Black caiman* and *Spectacled caiman* and other crocodilian species.

FA (%)	Lipids %[1]							
	C. crocodilus	*M. niger*	*p value*	*A. mississipiensis*[2]	*C. latirostris*[3]	*C. niloticus*[4]	*C. crocodillus yacare*[5]	
							Captivity	Wild
Fat % (DM[6])	0.02±0.00[a]	0.60±0.02[a]	0.0722	1.2±0.1	16.9±9.8	1.8±0.3	3.20	19.16
Lauric (12:0)	ND[7]	0.04±0.02[a]	0.0636	0.4	0.08	ND	NI[8]	NI
Myristic (14:0)	ND	ND	NA[9]	1.6	2.31	0.3 ± 0.1	NI	NI
Myristoleic (14:1)	ND	0.03±0.01[a]	0.0594	0.9	0.3	ND	NI	NI
Pentadecylic (15:0)	ND	ND	NA	1.1	0.55	0.1 ± 0.0	NI	NI
Palmític (16:0)	0.12±0.03[a]	1.41±0.06[a]	0.1000	17.5	21.85	20.2±0.1	NI	NI
Palmitoleic (16:1)	0.01±0.01[a]	0.56±0.03[a]	0.0765	5.3	2.72	3.1±0.3	3.93	5.9
Margaric (17:0)	ND	ND	NA	0.3	1.07	0,1±0,0	NI	NI
Heptadecanoic (17:1cis 10)	ND	0.05±0.01[a]	0.0594	NI	0.82	ND	NI	NI
Stearic (18:0)	0.06±0.02[a]	0.58±0.03[a]	0.1000	7.7	15.36	7.9±0.4	14.31	9.61
Vaccenic (18:1cis7)	ND	ND	NA	NI	NI	2.6±0.2	NI	NI
Oleic (18:1cis9)	0.05±0.02[a]	1.42±0.11[a]	0.1000	28.8	34.92	27.3±2.1	NI	NI
Linoleic (18:2 *n*-6)	0.02±0.01[a]	0.29±0.01[a]	0.0765	16.1	8.4	29.6±0.3	8.34	12.15
α-Linolenic (18:3 *n*-3)	ND	ND	NA	5.5	3.32	1.6±0.1	0.95	3.18
γ-Linolenic (18:3 *n*-6)	ND	ND	NA	NI	NI	0.2±0.1	0.42	0.58
Elaidic (18:1t9)	<0.01[a]	0.03±0.01[a]	0.0594	NI	NI	0.1±0.0	NI	NI
Arachidic (20:0)	<0.01[a]	0.01±0.01[a]	0.5050	0.3	NI	0.3 ±0.0	NI	NI
Eicosenoíc (20:1cis11)	<0.01[a]	0.06±0.02[a]	0.0636	NI	0.07	0.2 ± 0.1	NI	NI
Eicosadienoíc (20:2cis11,14)	<0.01[a]	0.02±0.01[a]	0.1876	NI	0.17	0.3±0.1	NI	NI
Eicosapentaenoic (20:5 *n*-3)	<0.01[a]	0.03±0.01[a]	0.0594	NI	0.76	0.2 ± 0.1	0.36	0.21
Eicosatrienoic (20:3)	0.04±0.01[a]	0.02±0.01[a]	0.1642	0.2	NI	042 ± 0.1	NI	NI
Arachidonic (20:4 *n*-6)	ND	ND	NA	2.9	4.34	4.2 ± 0.1	7.2	6.24
Docosapentaenoic (22:5 n-3)	ND	ND	NA	1.6	NI	0.5 ± 0.4	NI	NI
Docosahexaenoic (22:6 *n*-3)	ND	ND	NA	2.3	0.57	1.1 ± 0.3	0.69	1.69

[1]Values presented as: mean±standard deviation; numbers followed by the same letter are not significantly different; [2] Staton et al. (1990): sample of muscle tissue of captivity animals fed with mixed oils; [3] Cossu et al. (2007): intramuscular captivity animals; [4] Osthoff et al. (2010) samples of muscle of animals from captivity; [5] Vicente Neto et al. (2010) samples of muscle of animals from captivity and natural habitat; [6] Dry matter; [7] Not Detected; [8] Not Informed; [9] Not Applicable

over time (seasonal). At the time of sampling (high water level season), the animals may have less availability of food, as previously reported by Da Silveira and Magnusson (1999). Romanelli and Schmidt (1999) reported average fat ranging from 22 to 52% in caiman meat products, such as meat flour from caiman's viscera. These values show that the flour contains fat body, found in the coelomic cavity within the viscera and shows accumulation of fat within the abdominal cavity. On the other hand, Paulino et al. (2011) found lipid content ranging from 6.27 to 11.47% in different formulations prepared to make hamburgers with yacare meat using meat residue resulting from the deboning of the feet, back, and

tail of this alligator species. Romanelli et al. (2002) developed a product similar to canned meat using yacare meat from muscles of the trunk. The average lipid content of this product was 5.5%. The authors also developed a smoked meat product (raw cured meat from the tail) with an average lipid content of 5.36%. A lipid content of 12.8% was found in broad-snouted caiman meat preserved in oil (due to the addition of oil); whereas, contents of 2.4% were obtained in this meat preserved in onions and 1.4% when preserved in different seasonings and spices (Azevedo et al., 2009).

Several studies have been published on the characterization of crocodilian fat. Huchzermeyer (2003) reported two types of fats in crocodilians: (a) somatic fat, fat stored in the somatic cells with a small nuclei (in the thorax mediastinum, under the peritoneum and between muscles, the inner (caudofemoralis) and external (ilioischiocaudalis) muscles) and (b) fat body (within the coelomic cavity). The fat body content of crocodilians appears to vary according to age, gender, season, food availability, and animal origin (captive or wild) (Huchzermeyer, 2003). Other studies have reported that the fat content of animals bred in captivity under controlled environmental conditions and controlled food access is different from that of free-living caimans, which are exposed to larger seasonal variation. Concerning the lipid content of commercial cuts of other crocodilian species, some previous work reported levels about 4.39% in *Caiman* sp. (Cossu et al., 2007); 8.8% in *C. niloticus* (Hoffman et al., 2000); 1.9% in *Crocodilus porosus* (RIIRDC, 2007); and 1.5% in *Alligator mississipiensis* (Moody et al., 1980).

The analytical results of FA were not detectable (below the limit of detection (LOD) of <0.01) for most of the spectacled caiman samples assessed. The FA detected were: palmitic acid (0.12%), palmitoleic acid (0.01%), stearic acid (0.06%), oleic acid (0.05%), and eicosatrienoic acid (0.04%). SFA were present in larger amounts than those of PUFA and MUFA in the two species evaluated, with higher contents of palmitic acid (1.41%) and stearic acid (12.58%) in the black caiman. Vicente Neto et al. (2010) also found high content of stearic acid in the yacare caiman, both in captive and in wildlife animals, 9.61 and 14.31%, respectively. The ω-3 fatty acid (ALA and DHA) contents were below the LOD in the species evaluated; however, an EPA content of 0.03% was found in the black caiman only. As for the ω-9 FA family, the oleic acid was predominant, but there were no statistical significant differences in its content between the black caiman and spectacled caiman, 0.05 and 1.42% ($p = 0.1000$), respectively. It is worth to highlight the importance of this EFA in human nutrition as well as its successful clinical application in the prevention of cardiovascular diseases (Wang et al., 2006). In another species of amazonian fish with commercial exploitation, “pirarucu” or arapaima (*Arapaima sp.),* SFA content of 1.76% and 0.18% of PUFA was found (Scherr et al., 2014). In the captive-bred animals of the species *A. mississipienssis, C. latirotris,* and *C. niloticus,* studied by other authors, there were several FA present in undetectable amounts. Vicente Neto et al. (2010) determined the major FA present in the meat of *C. yacare*, specifically in neck and tail cuts. These authors found that the PUFA content was higher for animals in their natural habitat (31.0%) than for those in captivity (23.6%). Peplow et al. (1990) evaluated the FA profile in captive-bred *A. mississipienssis* and found differences in the FA contents between animals from different areas. According to these authors, a fish-based diet greatly influences the FA profile, showing higher amounts of eicosanoic acid than those found in animals fed meat diet. Staton et al. (1989) stated that *A. mississipienssis* fed diet with lower FA content and showed lower growth rate and that a diet with arachidonic acid appeared to enhance the growth rate of these animals. In another study, Mitchell et al. (1995) found high contents of oleic acid (33.0%), palmitic acid (22.5%), and linoleic acid (15.2%) in the meat of *C. porosus* and *Crocodilus johnstoni.* According to Cossu et al. (2007) there is a ω-3/ω-6 ratio (3.16) in the tail of caimans *(C. latirostris* and *C. yacare)*, near the optimum of 4 recommended by United States Department of Agriculture (USDA) (2006). In the present study, although the presence of ω-3 and ω-6 FA was detected in the commercial cut (tail fillet), their low amounts do not meet official health requirements.

Another important factor to be considered is that the seasonal variation influences the lipid composition of Amazonian fish species. Almeida et al. (2008) studied the FA profile in the muscle, orbital cavity, and abdominal cavity of “tambaqui” or black pacu (*Colossoma macropomum*), wild and in captivity, in different times of the year. The authors concluded that the FA profile of the free-living fish is more adequate for human consumption and that the animals caught during the dry season had higher amounts of PUFA.

Table 2 shows the FA profile in the fat body and somatic fat in *M. niger* samples. With the exception of the margaric acid and elaidic acid ($p \leq 0.0403$), all others showed normality in their frequency distribution ($p \geq 0.0502$). There was homogeneity of variance between the FA groups ($p \geq 0.0538$). However, when comparing the FAs, statistical significant differences were observed only in the contents of stearic acid, arachidic acid, and eicosanoic acid ($p \leq 0.0440$). Among the EFAs, the DHA content found in the fat tissues evaluated was of 0.88% (fat body) and 0.75% (somatic fat). Nevertheless, the amounts found the in the samples evaluated are not significant when compared with those reported by Osthoff et al. (2010), who found an average content of 9.4% in wild animals (*C. niloticus*). When comparing this DHA value with those of beef and chicken, the most widely eaten meats in Brazil, it is clear that the values found in *M. niger* are higher since Daley et al. (2010) reported a

Table 2. Fat acid profile in lipids samples from *Melanosuchus niger.*

FA (%)	Lipids (%)[1]			*C. niloticus*[2]
	Fat body	Somatic	*p value*	
Lauric (12:0)	0.13±0.03[a]	0.11 ±0.05[a]	0.4732	0.11 ±0.0
Myristic (14:0)	2.06±0.31[a]	1.56±0.49[a]	0.1026	3.9 ±0.3
Myristoleic (14:1)	0.49±0.07[a]	0.39±0.12[a]	0.1365	0.1 ±0.0
Pentadecyclic (15:0)	1.78±0.39[a]	1.30±0.37[a]	0.1044	0.3 ±0.1
Cis-10-pentadecanoic (15:1)	0.09±0.01[a]	0.09±0.02[a]	0.5556	NI[3]
Palmitic (16:0)	9.71±5.08[a]	4.41±4.28[a]	0.1411	25.6 ±1.6
Palmitoleic (16:1)	5.22±0.44[a]	4.98±1.31[a]	0.7107	6.2 ±0.3
Margaric (17:0)	0.92±0.78[a]	0.82±0.57[a]	0.4127	0.5 ±0.1
Heptadecenoic (17:1)	0.57±0.09[a]	0.49±0.12[a]	0.2653	ND[4]
Stearic (18:0)	5.00±0.62[a]	3.73±0.95[b]	*0.0440	4.7 ±1.0
Elaidic (18:1n9trans)	0.39±0.12[a]	0.33±0.16[a]	0.2857	0.1 ±0.1
Oleic (18:1cis9)	8.62±0.72[a]	7.43±1.50[a]	0.1592	28.0 ±1.8
Linoleic (18:2 *n*-6)	2.83±0.52[a]	2.58±0.86[a]	0.6030	6.5 ±2.6
Araquidic (20:0)	0.31±0.05[a]	0.21±0.06[b]	* 0.0261	0.3 ±0.0
γ-Linolenic (18:3cis3 *n*-6)	0.16±0.03[a]	0.15±0.05[a]	0.7141	0.2 ±0.0
Linolenic (18:3 *n*-3)	1.86±0.47[a]	1.68±0.63[a]	0.6375	2.0 ±0.4
Eicosenoíc (20:1cis11)	0.60±0.16[a]	0.39±0.09[a]	0.0590	0.5 ±0.2
Heneicosanoic (21:0)	0.13±0.02[a]	0.08±0.02[b]	* 0.0123	NI
Eicosadienoic (20:2)	0.28±0.03[a]	0.21±0.04[b]	* 0.0290	1.9 ±2.2
Behenico (22:0)	0.20±0.04[a]	0.14±0.05[a]	0.0541	0.1 ±0.0
Eicosatrienoic (20:3)	0.37±0.03[a]	0.31±0.07[a]	0.1170	0.3 ±0.2
Arachidonic (22:1)	0.07±.0.01[a]	0.05±0.02[a]	0.1372	0.8 ±0.3
Docosahexaenoic (22:6 *n*-6)	0.88 ±0.8[a]	0.75 ±0.24[a]	0.3114	9.4 ±1.9

[1]Values presented as: mean±standard deviation; numbers followed by the same letter are not significantly different; *FA with higher amount among the evaluated samples; [2]Osthoff et al. (2010) samples of adipose fat from wild animals (*C. niloticus*); [3]Not Informed; [4]Not detected

DHA content in beef of 0.20%. On the other hand, the DHA content in chicken breast was reported as 0.04% (Mirghelenj et al., 2009). Therefore, the results obtained in the present study are relevant since DHA is essential in the diet and cannot be synthesized in the human body and, like the ALA and EPA acids, it is considered as a functional substance. Nevertheless, although the DHA has been more frequently studied in cold water fish, such as salmon and anchovy (Oksuz and Özyılmaz, 2010), its presence in tropical fish suggests the need for further studies on its use in food industries.

The fat body showed higher concentration of palmitic acid (9.71%) than that of somatic fat and muscle tissue, followed by oleic acid (8.62%). This value can be explained because the steatotheca (abdominal fat body) is located in the mesenteric fold close to the abdominal wall and its amount varies depending on the nutritional status, while its shape varies in different species. Fat cells have wide nuclei able to pick up stored fat quickly. Osthoff et al. (2014) studied different adipose tissues of *C. niloticus* and found no statistically significant differences between the stheatoteca and the abdominal tissue. They reported that males have higher SFA content (44.4%) than that of PUFA and MUFA. Somatic fat had higher content of oleic acid (7.43%) than that of other FA, followed by palmitoleic acid (4.98%). Similarly, Almeida and Franco (2007) found SFAs in another fish species, wild “matrinxã” (*Brycon cephalus*). On the other hand, the PUFA content in wild animals was higher than that of captive-bred animals, including DHA. Castelo (1981) studied the FA composition in the species “piracatinga” or red-bellied pacu (*Colossoma bidens*) and “pacu-caranha” or pacu (*Colossoma mitrei*) and found that the oleic acid was predominant in the two species with 44.48 and 48.71%, respectively. These authors stated that the content of fat within the abdominal cavity is dependent on seasonal food consumption, which can even change the fat color from light yellow to dark yellow. Like the “tambaqui” or black pacu, these species are omnivorous and feed on various fruits and vegetables, which can explain why these fats are more similar to vegetable oils than are to saltwater fish oil. Almeida and Franco (2006) also reported that palmitic acid and oleic acid are the most predominant FAs in freshwater fish, which is in accordance with the results of FA content obtained in the present study. Additionally, the intake of

FAs through amazonian fish consumption has clinical benefits. Souza et al. (2002) investigated the addition of “tambaqui” fat in laboratory animals and concluded that it is a good dietary source of lipids and can substitute beef fat producing effects similar to those of soybean oil when risk factors for atherosclerotic are considered.

Conclusion

The variability of FAs found in the lipid profile of crocodilians studied is significant from nutritional and commercial point of view because it suggests the possibility of obtaining high calorie products. It was found that the average content of lipids in the fats and commercial cut studied strongly contributes to the total caloric value of the caiman meat, making it a nutritionally attractive product. On the other hand, in future works, it is important to collect the samples in the low water season to evaluate if there are changes in the FA profile. Considering that some PUFAs with clinical significance were present in the samples investigated, it would be important to assess their effect on consumers’ diet, as the FA absorption in humans varies according to the microbiome. Therefore, an *in vivo* study on the properties of Amazon caiman’s fat and its antioxidant activity is suggested to investigate its possible biotechnological use as nutraceutical, such as the fish oils that have already been manufactured by the pharmaceutical companies.

Conflict of interest

The authors have not declared any conflict of interest.

AKNOWLEDGEMENTS

FAPEAM Amazonas State Research and Technology Suport Fundation. Field data collection was financed by Ministério da Ciência, Tecnologia e Inovação (MCTI/ Brasil), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) through the BAJAQUEL Project (408760/2006-0 granted to Ronis Da Silveira).

REFERENCES

Almeida NM, Visenteiner JV, Franco MRB (2008). Composition of total, neutral and phospholipids in wild and farmed tambaqui (*Colossoma macropomum*) in the Brazilian Amazon area. J. Sci. Food Agric. 88:1739-1747.

Almeida NM, Franco MRB (2007). Fatty acid composition of total lipids, neutral lipids and phospholipids in wild and farmed matrinxã (*Brycon cephalus*) in the Brazilian Amazon area. J. Sci. Food Agric. 87:2596-2603.

Almeida NM, Franco MRB (2006). Influence of fish feeding diet on its fatty composition: nutritional aspects and benefits to human health. Rev. Inst. Adolfo Lutz 65:7-14.

AOAC (2005). Official methods of analysis (17th ed.). Gaithersburg, MD, USA: AOAC International.

Azevedo IC, Carmo RP, Torres AG, Marsico EI, Freitas MQ (2009). Acceptance test and percent composition of broad-snouted caiman (*Caiman latirostris)* canned meat**.** Ciênc. Rural. 39:534-539.

Bauer DF (1972). Constructing confidence sets using rank statistics. J. Am. Stat. Assoc. 67:687-690.

Canto ACVCS, Lima BRCC, Cruz AG, Lazaro CA, Freitas DGC, Faria JAF, Torrezan R, Freitas MQ, Silva TPJ. (2012). Effect of high hydrostatic pressure on the color and texture parameters of refrigerated Caiman (*Caiman crocodilus yacare*) tail meat. Meat Sci. 91:255-260.

Castelo FP (1981). Características da gordura cavitárias de pirapitinga, *Colossoma bidens* e pacu-caranha (*Colossoma mitrei*). Acta Amazonica. 11:255-265.

Cernıkova M, Gal R, Polasek Z, Janıcek M, Pachlova V, Bunka F (2015). Comparison of the nutrient composition, biogenic amines and selected functional parameters of meat from different parts of Nile crocodile (*Crocodylus niloticus*). J. Food Comp. Anal . 43:82-87.

Conover WJ, Johnson ME, Johnson MM (1981). A comparative study of tests for homogeneity of variances, with applications to the outer continental shelf bidding data. Technometrics 23:351-361.

Cossu ME, Gonzalez OM, Wawrzkiewicz, M, Moreno D, Vieite CM (2007). Carcass and meat characterization of “Yacare overo” (*Caiman latirostris*) and “Yacare negro” (*Caiman yacare*). Braz. J. Vet. Res. Anim. Sci. 44:329-336.

Da Silveira R, Magnusson WE (1999). Diets of Spectacled and Black Caiman in the Anavilhanas Archipelago, Central Amazonia, Brazil. J. Herpetol. 33:181-192.

Da Silveira R, Thorbjarnanarson J (1999) Conservation implications of commercial hunting of black and spectacled caiman in the Mamirauá Sustainable Development Reserve, Brazil. Conservat. Biol. 88:103-109

Folch J, Lees M, Stanley GH (1957). A simple method for the isolation and purification of total lipids from animal tissue. J. Biol. Chem. 226:497-509.

Hartman L, Lago RC (1973). Rapid preparation of fatty acid methyl esters from lipids. Laboratory Pract. 22:475-476.

Hoffman LC, Fisher PP, Sales J (2000). Carcass and meat characteristics of the Nile Crocodile (*Crocodylus niloticus*). J. Sci. Food Agric. 80:390-396.

Hollander M, Wolfe DA (1999). Nonparametric Statistical Methods. 2 ed. New York: John Wiley & Sons.

Kaur N, Chugh V, Gupta AK (2014). Essential fatty acids as functional components of foods- a review. J Food Sci. Technol. 51:2289-2303.

Kluczkovski Júnior A, Kluczkovski AM, Moroni FT, Markendorf F, Inhamuns A. (2015). Carcass Yield and composition of *Melanosuchus niger*. Intern. J. Fish. Aquact. 7:47-53.

Martin CA, Almeida VV, Ruiz MR, Visentainer JEL, Matshushita M, Souza NE, Visentainer JV (2006). Omega-3 and omega-6 polyunsaturated fatty acids: importance and occurrence in foods. Rev. Nutr. 19:761-770.

Mirghelenj SA, Golian A, Taghizadeh V (2009). Enrichment of chicken meat with long chain omega-3 fatty acids throught dietary fish oil. Res. J. boil. Sci. 4:604-608.

Mitchell GE, Reed AW, Houlihan DB (1995). Composition of crocodile meat (*Crocodylus porosus* and *Crocodylus johnstoni*). Food Australia*.* 47:221-224.

Moreira NX, Curi R, Mancini FJ (2002). Fatty acids: a review. Nutrire. 24:105-123.

Ohr L M (2005). Functional fatty acids. Food Technol. 59:63-65.

Öksüz A, Özyılmaz A (2010). Changes in Fatty Acid Compositions of Black Sea Anchovy (*Engraulis encrasicolus* L. 1758) During Catching Season. Turk. J. Fish. Aq. Sci. 10:381-385.

Osthoff G, Hugo A, Govender D, Huchzermeyer F, Bouwman H (2014). Comparison of the lipid composition of three adipose tissue types of male and female wild Nile crocodiles (*Crocodylus niloticus*). J. Herpetol. 48:525-531.

Osthoff G, Hugo A, Bouwman H, Buss P, Govender D, Joubert CC, Swarts JC (2010). Comparison of the lipid properties of captive, healthy wild and pansteatitis-affected wild Nile crocodiles (*Crocodylus niloticus*). Comp. Biochem. Physiol. 155:64-69.

Paulino FO, Silva TJP, Franco RM, Marsico ET, Canto ACVC, Vieira JP, Amaral AP, Pereira AAS (2011). Processing and quality

characteristics of hamburger of Pantanal alligator meat (*Caiman crocodillus yacare*). Rev. Bras. Cienc. Vet. 18:129-132.
Peplow A, Balaban M, Leak F. (1990). Lipid composition of fat trimmings from farm-raised alligators. Aquaculture 91:339-348.
R Core Team. (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org.
Razali N, Wah YB (2011). Power comparisons of Shapiro-Wilk, Kolmogorov-Smirnov, Lilliefors and Anderson-Darling tests. J. Stat. Modeling Anal. 2:21-33.
RIIRDC (2007). Composition of new meats, analyses and nutrient composition of innovative meat industries. Rural Industries Research and Development Corporation Publication N. 07/036, Australia.
Romanelli PF, Felicio PE (1999). Jacaré do Pantanal (*Caiman crocodilus yacare*): Rendimentos de abate e composição da carne. Higiene Alimentar. 13:11-15.
Romanelli PF, Caseri R, Lopes Filho JF (2002). Meat processing of pantanal alligator (*Caiman crocodilus yacare*). Ciênc. Tecnol. Aliment. 22:70-75.
Ross JP (2000). *Melanosuchus niger*. The IUCN Red List of Threatened Species 2000: e.T13053A3407604. http://dx.doi.org/10.2305/IUCN.UK.2000.RLTS.T13053A3407604.en.
Russo GL (2009). Dietary n-6 and n-3 polyunsaturated fatty acids: From biochemistry to clinical implications in cardiovascular prevention. Biochem. Pharmacol. 77:937-946.
Scherr C, Gagliardi ACM, Miname MH, Santos RD (2015). Fatty Acid and Cholesterol Concentrations in Usually Consumed Fish in Brazil. Arq. Bras. Cardiol. 104:152-158.
Simopoulos A (2016). An Increase in the Omega-6/Omega-3 Fatty Acid Ratio Increases the Risk for Obesity. Nutrients 8(3):128.
Simopoulos A (2008).The omega-6/omega-3 fatty acid ratio, genetic variation, and cardiovascular disease. Asia Pac. J. Clin. Nutr. 17:131-134.
Siro I, Kapolna E, Kapola B, Lugasi A. (2008). Functional food. Product development, marketing and consumer acceptance--a review. Appetite. 51(3):456-467.
Souza RV, Santos PCF, Bambirra EA, Vieira EC, Alvarez-Leite JI (2002). Nutritional Characteristics of Amazonian Fish Fat (*Colossoma macropomum*) and its effect on lipid metabolism of rats fed hypercholesterolemic diets. Ciênc. Tecnol. Aliment. 22:88-93.
Staton MA, Edwards Jr HM, Brisbih Jr IL, Joaneh T, Mchease L (1990). Fatty Acid Nutrition of the American Alligator (*Alligator mississipiensis*). J. Nutr. pp. 674-685.
USDA. US Department of Agriculture (2006). Agricultural Research Service, Nutrient Data Laboratory. USDA National Nutrient Database for Standard Reference, Release 19. Washington, DC: USDA.
Vicente Neto J, Bressan MC, Rodrigues EC (2007). Avaliação físico química da carne de jacaré-do-Pantanal (*Caiman yacare* Daudin 1802) de idades diferentes. Ciênc. agrotec. 31:1430-1434.
Vicente Neto J, Bressan MC, Faria PB, Vieira JO, Santana MTA, Kloster M (2006). Composição centesimal e colesterol da carne de jacaré-do-Pantanal (*Caiman yacare* Daudin 1802) oriundo de zoocriadouro e habitat natural. Ciênc. Agrotéc. 30:701-706.
Vicente Neto J, Bressan MC, Faria PB, Vieira JD, Cardoso MG, Glória MBA, Gama LT (2010). Fatty acid profiles in meat from *Caiman yacare* (*Caiman crocodilus yacare*) raised in the wild or in captivity. Meat Sci. 85:752-758.
Wang C, Harris WS, Lichtenstein AH, Balk EM, Kupelnick B, Jordan HS, Lau J (2006). n-3 Fatty acids from fish or fish-oil supplements, but not α-linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review 1–3. Am. J. Clin. Nutr. 84:5-17.

academicJournals

Vol. 15(29), pp. 1566-1574, 20 July, 2016
DOI: 10.5897/AJB2016.15222
Article Number: D2F94C959635
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Osmotic stress upregulates the transcription of thiamine (vitamin B1) biosynthesis genes (THIC and THI4) in oil palm (*Elaies guineensis*)

Wong Sook Yee, Syamimi Diyana Abdul Aziz and Zetty Norhana Balia Yusof*

Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

Received 12 January, 2016, Accepted 11 July, 2016.

Thiamine or vitamin B_1 comprises a pyrimidine moiety and a thiazole moiety. Thiamine pyrophosphate (TPP), the active form of thiamine, acts as a cofactor for various major enzymes, for example, transketolase (TK), α-ketoglutarate dehydrogenase (KGDH) and pyruvate dehydrogenase (PDH). It is suggested that TPP plays another important role, which is protecting plants against abiotic and biotic stresses such as osmotic stress. In this study, the gene transcripts of first two enzymes in thiamine biosynthesis pathway, THIC and THI1/THI4 were identified and amplified from oil palm tissues. Primers were designed based on sequence comparison of the genes from *Arabidopsis thaliana*, *Zea mays*, *Oryza sativa* and *Alnus glutinosa*. Oil palm's responses in terms of the expression profiles of these two thiamine biosynthesis genes to an osmotic stress inducer, polyethylene glycol (PEG) were examined. The level of gene transcripts was analyzed using reverse transcriptase polymerase chain reaction (RT-PCR) and both THIC and THI1/THI4 gene transcripts were successfully amplified. The levels of transcription were measured and the results showed that the expression of THIC gene transcripts showed an increase of up to 200% in 1% PEG treated plant as compared to non-treated plant while the expression of THI1/THI4 gene transcripts showed an increase of up to 100% in treated plant as compared to non-treated plant. However, increase in concentration of PEG showed decrease in amount of transcription level for both gene transcripts. The results support the suggestion that thiamine may play an important function in plant defense against stresses as these findings may lead to an overexpression of thiamine in general.

Key words: Thiamine, vitamin B1, osmotic stress, oil palm, gene expression.

INTRODUCTION

Thiamine is important for all living organism as it serves vital functions in carbohydrate metabolism, nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP) synthesis and also

*Corresponding author. Email: zettynorhana@upm.edu.my.

nucleic acids formation (Nosaka, 2006). Thiamine pyrophosphate (TPP), the active form of thiamine acts as a cofactor for various key enzymes for example pyruvate dehydrogenase, transketolase and α-ketoglutarate dehydrogenase (Frank et al., 2007). Thiamine and its derivative, thiamine pyrophosphate (TPP) play important roles in human nutrition and central metabolism (Tunc-Ozdemir et al., 2009; Guan et al., 2014). In previous studies, thiamine and TPP are reported as crucial stress-response molecules in plant adaptations to counteract different abiotic stress conditions (Tunc-Ozdemir et al., 2009; Rapala-Kozik et al., 2012). Thiamine enhances resistance to oxidative stress via salicyclic acid (SA) signaling pathway (Ahn et al., 2007).

Naturally, osmotic stress occurs through drought, salinity or even cold stress which lowers the water potential in plant cell (Hoffmann, 2002). Studies have shown that deficit in water status in plants has led to the rapid changes in gene expression (Yamaguchi-Shinozaki and Shinozaki, 2006; Osakabe et al., 2011). Besides that, osmotic stress in plants has been shown to inhibit the plant growth via efflux of potassium ion (K^+) which is essential for osmoregulation and plant growth (Maggio et al., 2006; Zonia and Munnik, 2007). K^+ aid in guard cells' functions in opening and closure of stomata for osmoregulation (Schroeder and Hagiwara, 1989; Pei et al., 1997; Ache et al., 2000; Hosy et al., 2003; Kim et al., 2010) and regulates the sap flow from roots to shoots in plants with nutrients for plant growth (Lebaudy et al., 2007). Upregulation of phytohormone synthesis, such as abscisic acid, under osmotic stress has also been reported (Osakabe et al., 2013). Apart from that, osmotic stress has also showed evidence in formation of oxygen radical species via the upregulation of antioxidant enzymes and accumulation of compounds which neutralize those radical species (Upadhyaya and Panda 2004; Upadhyaya *et al*., 2008, 2011; Gill and Tuteza 2010). Severe exposure to osmotic stress to plants will lead to wilting and necrosis (Upadhyaya et al., 2013).

Thiamine biosynthesis

Animals and humans must consume thiamine through their diets because they cannot synthesize it, while plants and microorganism can biosynthesize it *de novo* (Moulin et al., 2013). The pyrimidine and thiazole moieties are synthesized in distinct branches of the pathway and are then condensed to form thiamine monophosphate (TMP). TMP is then phosphorylated by a specific kinase to form TPP. Plants synthesize TPP from elementary precursors via *de novo* biosynthetic pathways that are analogous to both bacteria and yeast (Goyer, 2010). The initial phases of TPP biosynthesis include two parallel pathways. One is like the mechanism found in bacteria in which the pyrimidine branch of thiamine (4-amino-2-methyl-5-hydroxymethylpyrimidinemonophosphate, HMP-P) is synthesized from 5-aminoimidazole ribonucleotide (AIR) and is catalysed by an enzyme, which is encoded by THIC gene and has been identified in *Arabidopsis thaliana* (Raschke et al., 2007). The other pathways are similar to the mechanism found in yeast (Chatterjee et al., 2008) wherein the thiazole branch of thiamine (4-methyl-5-(2-hydroxyethyl)-thiazole phosphate, HET-P) is synthesized from glycine, nicotinamide adenine dinucleotide (NAD^+) and a sulphur donor protein. The THI1 gene, which has been identified in *Zea mays* and *Arabidopsis thaliana* (Machado et al., 1996) and its orthologue, the THI4, which is found in bacteria encodes for the main enzyme that synthesizes HET-P. Studies on THI4 gene in yeast proved that it has two functions, in thiamine biosynthesis and also in DNA damage tolerance when subjected to abiotic stress (Machado et al., 1997). Research by Rapala-Kozik et al. (2008) revealed the regulation of thiamine metabolism in *Z. mays* seedlings under different abiotic stresses. In the study, *Z. mays* seedlings were exposed to drought, salinity and oxidative stress. The total thiamine content in the maize seedlings increased under different stress conditions. Experiments by Tunc-Ozdemir et al. (2009) proved that under oxidative stress, high salinity, sugar deprivation and hypoxia, the regulation of THI1 gene transcript was increased. Thiamine and TPP serve as essential stress-response molecules during different abiotic stress conditions to combat the condition. Accumulation of thiamine and TPP was observed in *Arabidopsis* when exposed to abiotic stresses like high light intensity, low temperature, osmotic, salinity and oxidative treatment. Enhanced tolerance towards oxidative stress was observed when plants were supplemented with exogenous thiamine. Studies by Ahn et al. (2005) and Zhang et al. (1998) also showed that thiamine affects the defence-related genes or systemic acquired response (SAR)-related genes expression in plants for example tobacco, *Arabidopsis* and wheat. The SAR-related genes were expressed enormously in thiamine treated plants. Recently, a study by Balia Yusof et al. (2015) proved that there is an upregulation in the expression of THIC and THI1/THI4 genes transcripts in *Ganoderma boninense* infected oil palm.

Since the previous studies have shown that thiamine can improve the immune system of plants, it is believed that sustained accumulation of thiamine can make plants immune to severe diseases. Oil palm is an economically valuable crop for its oil and has become one of the leading oil crops in the world. Malaysia, the world's second largest producer and exporter of palm oil and its by-products, produced nearly 18 million tons in 2011. However, as sessile organism, oil palm faces a whole lot of stresses. Both biotic and abiotic stresses have negative influences on oil palm survival, palm oil production and crop yield. Common environmental stresses in Malaysia include water deficit, high

temperature and salinity. Among the abiotic factors, water deficit is the most common stress that restricts oil palm growth, survival, distribution and productivity.

In this study, the changes in the transcription level of thiamine biosynthesis genes (THIC and THI1/THI4) in oil palm when subjected to polyethylene glycol (PEG)-induced osmotic stress was analyzed. Since thiamine may play an important function in plant protection against stress, it is hypothesized that there will be an increase in the transcription level of THIC and THI1/THI4 gene transcripts once subjected to osmotic stress.

MATERIALS AND METHODS

Plant materials and stress treatment

A total of 12 six-month old commercial Dura × Pisifera oil palm seedlings were obtained from Sime Darby, Banting. The palms were arranged in a shaded area using randomized complete block design (RCBD) and subjected to the general nursery practices. A set of 3 seedlings were used as control (0% PEG) and different concentrations of polyethylene glycol 6000 (PEG) were given as treatments (1% PEG, 3% PEG and 5% PEG) for its respective sets.

Sampling

Sampling was scheduled at 3, 7 and 30 days post-treatment. Spear leaves were taken as tissue samples from 3 seedlings at each sampling point. The spear leaves were cleaned and cut before being frozen in liquid nitrogen and kept at -80°C for further use.

Data mining and primer designing

Data mining includes the gathering of genes sequences of thiamine biosynthesis pathway (THIC and THI1/THI4) from different plant species from the GenBank of NCBI database. The nucleotide sequences were aligned using ClustalW program and the consensus regions were identified and used to design PCR primers.

Total RNA isolation and quantitation

Total RNA was isolated using the modified RNA extraction protocol by Li and Trick (2005). The tissues from spear leaves at 3-, 7- and 30- days post-treatment were used for RNA extraction. The assessment of RNA quality and integrity was done using a NanoPhotometer (Implen, Germany) and the purified RNA was stored at -80°C.

Amplification of THIC and THI1/THI4 genes

RT-PCR was performed using Tetro cDNA Synthesis Kit (Bioline, USA). Complementary DNA (cDNA) was prepared by mixing 5 µg/µl of total RNA, 4 µl of 5× Reverse Transcriptase Buffer, 1 µl of 10 mM dNTP mix, 1 µl of Oligo $(dT)_{18}$ Primer, 1 µl of Ribosafe RNase Inhibitor, 1 µl of Tetro Reverse Transcriptase and up to 20 µl of DEPC-treated water. The solution was mixed gently via pipetting prior subjection to incubation at 45°C for 30 min. The solution was then incubated at 85°C for 5 min to stop the reverse transcriptase reaction. The cDNA prepared was then chilled in ice and stored in -20°C until used for analysis with PCR.

PCR reaction was performed by using MyTaq™ Red Mix (Bioline, USA). A 25 µl reaction was prepared by mixing 1 µl of cDNA Template, 0.5 µl of forward primer, 0.5 µl of reverse primer, 12.5 µl of MyTaq Red Mix and up to 25 µl of deionized water. The reaction mixture was mixed and placed inside the thermocycler (Biometra, Germany). The PCR cycling requirements included the initial denaturation step which was set at 95°C for 2 min for 1 cycle, followed by 28 repetitive cycles of denaturation step at 95°C for 45 s, annealing step at 55°C for 45 s and extension step at 72°C for 1 min. The final cycle of extension was set at 72°C for 5 min for 1 cycle and then held at 4°C. The optimized annealing temperature for THIC primer F3 and THI1/THI4 primer F8 are 55°C while for THIC primer F2 is 48°C. The PCR product was then kept in the -20°C freezer for further use.

Analysis of PCR products

PCR products were analyzed using gel electrophoresis to detect amplification of gene fragments. The gel was run alongside actin gene fragment which acts as control. The bands intensities were calculated using ImageJ software (http://imagej.nih.gov/ij/).

PCR product purification

The PCR products were purified using FavorPrep™ Gel Purification Kit (Favorgen). 100 µl of PCR products were transferred in to a microcentrifuge tube and mixed with 500 µl of FADF buffer by vortexing for 12 min. The mixture was then transferred into FADF column in a collection tube and centrifuged for 30 s at 13,000 rpm. The flow-through was discarded and 750 µl of wash buffer was added into the FADF column and centrifuged for 30 s and further centrifuged for 3 min at 13,000 rpm. The column was then placed into a new microcentrifuge tube where the purified PCR product was collected by adding 30 µl of elution buffer to the centre of the column and centrifugeD for 2 min at 13,000 rpm after 2 min standing at room temperature. Each purified PCR product was analyzed with NanoDrop-spectrophotometer for DNA purity and concentration prior to sending them to 1st Base Company (http://base-asia.com/dna-sequencing-services) for sequencing.

DNA sequencing

Purified PCR products were sent for sequencing at 1st BASE DNA sequencing service (1st BASE, Singapore). The sequencing results were then analyzed using Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov) to verify the amplified gene fragments.

RESULTS

Amplification of gene transcripts

Primers were designed based on sequence alignments of different plant species as shown in Table 1. Table 2 shows the designed primers used for this study. Level of transcription was shown visually in Figure 2 by which both THIC and THI1/THI4 gene transcripts were successfully amplified in non-treated oil palm tissues using the designed primers. The fragment size for THIC amplified using primer F2 is 410 bp, while the fragment

Table 1. Data from selected plant species that were used for primer designing and their accession number from GenBank of NCBI database.

Gene	Data from GenBank	Accession no.
THIC	*Arabidopsis thaliana* phosphomethylpyrimidine synthase mRNA, complete cds	NM_001202705.1
	Arabidopsis thaliana phosphomethylpyrimidine synthase mRNA, complete cds	NM_128517.3
	Arabidopsis thaliana phosphomethylpyrimidine synthase mRNA, complete cds	NM_179804.2
	Zea mays clone 378320 thiamine biosynthesis protein thiC mRNA, complete cds	EU972242.1
	Oryza sativa Japonica Group Os03g0679700 (Os03g0679700) mRNA, complete cds	NM_001057432.1
THI1/THI4	*Arabidopsis thaliana* thiazole biosynthetic complete cds	NM_124858.3
	Arabidopsis thaliana Thi1 protein mRNA, complete cds	U17589.1
	Zea mays thiamine biosynthesis1 (thi1), mRNA	NM_001112226.1
	Oryza sativa Japonica Group mRNA for thiamine biosynthetic enzyme, complete cds, clone: 12YPR001	AB110170.1
	A. glutinosa mRNA for thiazole biosynthetic enzyme	X97434.1

Table 2. Primers designed for the amplification of THIC, THI1/THI4 and Actin gene transcripts.

Description	Primer's name	Sequences
Primers designed for THIC gene	THIC F1	5'- CATTCCTCTTACAGCAAAG -3'
	THIC R1	5'- GTGATGTGATCATAACCAGG -3'
	THIC F2	5'- CTTACAGCAAAGAGAATGAC -3'
	THIC R2	5'- GTGATGTGATCATAACCAGG -3'
	THIC F3	5'- GGGATCATAACTGAGGCGGG -3'
	THIC R3	5'- ATCAGCTCCCCACATGGTTG -3'
	THIC F4	5'- CATAACTGAGGCGGGCGATT -3'
	THIC R4	5'- ACATGGTTGCCCAACTTCCT -3'
Primers designed for THI1/THI4 gene	THI4 F5	5'- CATGACGGACATGATCAC –3'
	THI4 F6	5'- CTCTCTTCACCTCAACCATC -3'
	THI4 R6	5'- GCCAATACTCTTCAGCCTC -3'
	THI4 F7	5'- CCAGCTTTTGGCTCGTCCTA -3'
	THI4 R7	5'- GTCAACCGCACAATAGCGTC -3'
	THI4 F8	5'- GACGCTATTGTGCGGTTGAC -3'
	THI4 R8	5'- TCCGTCAATAGCATTCGGCA -3'

size for amplified using THIC primer F3 is 156 bp. The fragment size for THI1/THI4 amplified using primer F8 is 180 bp.

Sequencing analysis

Purified PCR products were sent for sequencing and the sequencing results were verified using BLAST. The verifications are based on the alignment identity and E-value score values where it determines the quality and similarity between the query sequence (sequencing result) and sequences available in the Genbank. The lower the E-value score, the higher the relevancy of the sequences are. However, the higher the identity score, the higher the similarity of the sequences are. Sequencing result of actin gene fragment showed 98% identity with *E. guineensis* actin (ACT1) mRNA, complete cds (Accession number: AY550991.1) with an E value score of $5e^{-57}$. The fragment size amplified by the designed primer was 135 base pairs. Apart from that, sequencing result of THIC gene fragment amplified using primer F2 showed 99% identity with *E. guineensis* phosphomethylpyrimidine synthase, chloroplastic (LOC105046270), transcript variant X10, mRNA (Accession number: XM_010924818.1) with an E value score of 0.0. The fragment size amplified by the primer was 371 bp. Sequencing result of THIC gene amplified using primer F3 showed 92% identity with *E. guineensis* phosphomethylpyrimidine synthase, chloroplastic

(LOC105046270), transcript variant X10, mRNA (Accession number: XM_010924818.1) with an E value score of $1e^{-72}$. The fragment size amplified by the primer was 201 bp. All the mentioned sequencing results showed a high value of identity and acceptable E-value score which significantly identified the amplified fragments. However, sequencing was unsuccessful for THI1/THI4 PCR products which may be due to low PCR product concentration.

Analysis of PCR products

The level of transcription of both THIC and THI1/THI4 genes in treated and non-treated samples were analyzed using ImageJ software. In theory, it is suggested that the higher the band intensities, the more the copy number of the respective gene present in the cDNA mixture which indicates high level of transcription activity. The difference in the level of transcription in the control and PEG-treated tissue samples for both gene transcripts could be visually observed as shown in Figure 3.

Three days after the application of 1% PEG, high transcription level of THIC gene transcript was observed where there was 200% increase as compared to the control (Figure 4). However, on day 7, an increased transcription level up to 65% was observed for THIC gene transcript as compared to the control. The level of transcription of THI1/THI4 gene transcript 3 days after application of 1% PEG showed similar results as THIC gene transcript, where it showed 100% increase as compared to the control, followed by an increment of just 57% on day 7 post-treatment and only up to 28% increase on day 30 post-treatment. The THIC and THI1/THI4 genes transcriptions level were also altered in the presence of 3% PEG with a day profile similar to that of 1% PEG stress condition but the increase were only up to 1.7-fold. The gene expression pattern in the presence of 5% PEG was somehow lower as compared to the other concentrations of PEG. In short, under osmotic stress, the highest gene transcription level for both THIC and THI1/THI4 gene transcripts were observed on day 7 post- treatment (1.2-fold).

DISCUSSION

The level of gene transcription of two thiamine biosynthesis genes namely THIC and THI1/THI4 in oil palm was compared between normal condition and osmotic stress. It is believed that stress-triggered damage affects the important pathways and therefore apparently need to upregulate the thiamine biosynthetic process which is necessary (Rapala-Kozik et al., 2008, Tunc-Ozdemir et al., 2009). This study evaluated the genes encoding for the biosynthesis of the pyrimidine and thiazole moieties of thiamine (THIC and THI1/THI4). In determining the upregulation or downregulation of the genes of interest, an osmotic stress inducer, PEG was used where oil palm seedlings were given various concentrations and tissue samples were collected at 3 time points.

The results obtained showed that there was an increase in THIC and THI/THI4 gene transcription in the earlier stage specifically at day 3 after exposure to stress. This indicated that thiamine was needed to be synthesized in order to combat the damaging effect of osmotic stress. This is not surprising as activation of thiamine biosynthesis in plants is well established in response towards abiotic stresses (Ribeiro et al., 2005; Rapala-Kozik et al., 2012).

However, the decrease of the transcription level of THIC and THI1/THI4 gene transcripts along the period of stress treatment could be due to the fact that the palms are starting to adapt to the stress conditions. There is yet no proof that thiamine being directly involved in combating stresses, is known to have major roles in carbohydrate catabolism, NADPH and ATP synthesis and in the formation of nucleic acids (Rapala-Kozik et al., 2012) which are indirectly involved in the production of compounds involved in defence mechanism. Results from this study showed that the level of transcription for both gene were highly increased as compared to control up to day 7 post-treatment where it indicates thiamine was urgently needed. The decrease of the level of transcription of thiamine biosynthesis genes (same level with control) after day 7 probably showed the less need for thiamine due to the synthesis of stress-combating molecules which leads to the reduction of stress affects. This may show that hormonal signaling mechanism is responsible rather than water availability that affect transcriptional regulation under osmotic stress (Munns et al., 2000).

Apart from that, level of THIC gene transcript was observed to be higher as compared to THI1/THI4 gene transcript level. As previously mentioned, THIC and THI1/THI4 are the genes that encode the first enzymes of pyrimidine and thiazole moieties respectively of the thiamine biosynthesis pathway as shown in Figure 1. These enzymes play a crucial role in thiamine biosynthesis, yet they seem to have a non-cofactor function in DNA damage tolerance induced by abiotic and biotic stresses in plants (Goyer, 2010). Studies on THI4 gene in yeast have proved that it has a dual role, in thiamine biosynthesis and also in DNA damage tolerance when subjected to abiotic stress (Machado et al., 1997).

This may be the reason why somehow the upregulation of the THI1/THI4 gene transcript level was not as high as THIC gene. As a whole, the results showed that indeed, the transcription level for both genes transcript were upregulated in PEG-treated oil palm seedlings which proved this study hypothesis.

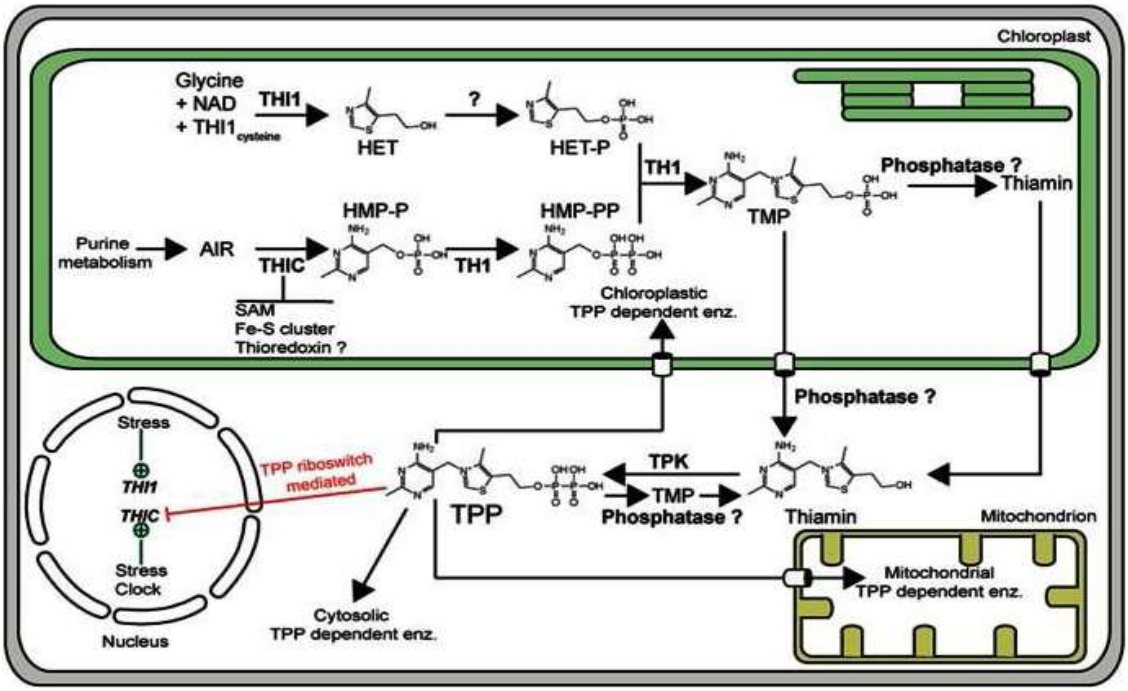


Figure 1. Thiamine biosynthesis pathway of *A. thaliana*. TMP is produced by the condensation of HMP-PP and HET-P. To form the active cofactor, TPP, TMP is first dephosphorylated by a phosphatase and then subsequently pyrophosphorylated by TPK (Pourcel et al., 2013).

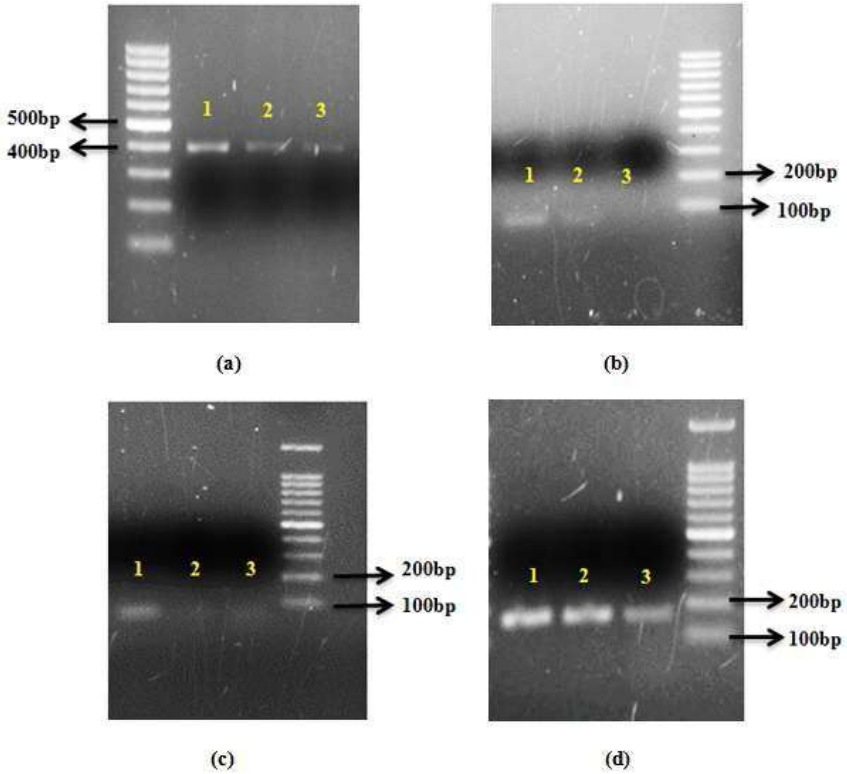


Figure 2. Amplification of (a) THIC primer F2; (b) THIC primer F3; (c) THI1/THI4 primer F8; (d) Actin from non-treated oil palm. Lane 1: spear leaf 3-days; lane 2: spear leaf 7-days; lane 3: spear leaf 30-days post-treatment.

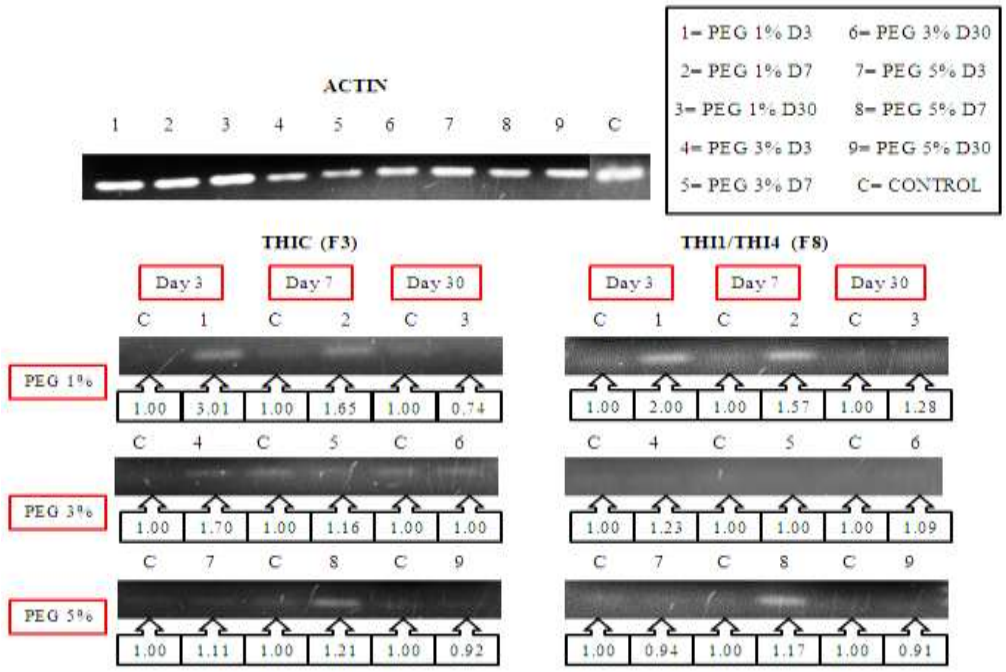


Figure 3. The level of gene expression for THIC and THI1/THI4 in treated and non-treated oil palm spear leaf tissue samples analyzed using ImageJ software. Lane C represents non-treated oil palm spear leaf tissue sample while lane 1-9 represent treated oil palm spear leaf tissue samples.

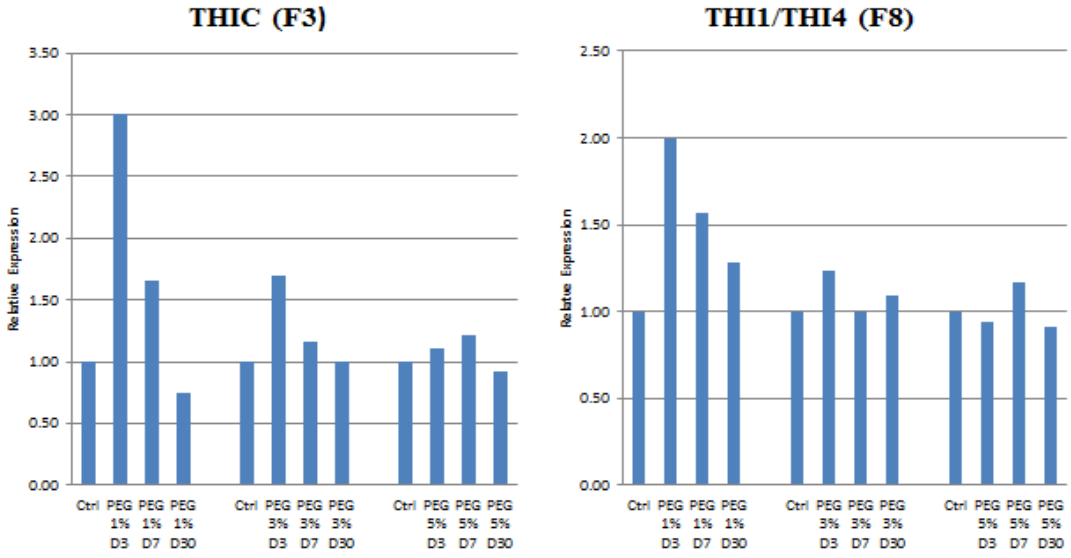


Figure 4. Day-dependent response of oil palm seedlings to osmotic stress revealed through THIC and THI1/THI4 gene expression changes.

Conclusion

Overall, in response to stress, oil palm showed an increase in the level of gene transcripts of the first two enzymes in the thiamine biosynthesis pathway. The results proved that thiamine biosynthesis genes, namely THIC and THI1/THI4, were upregulated under osmotic stress. An increase of transcription is therefore needed

to maintain the proper thiamine cellular levels as it is crucial to develop new adaptation strategies and to overcome the impacts of stress. These suggest that thiamine biosynthesis in plants is tightly modulated during stress sensing and adaptation.

However, this is a preliminary study which only involves thiamine biosynthesis study at transcriptional level. For future work, total thiamine content determination via HPLC analysis should be carried out to confirm the increase in thiamine accumulation. Apart from that, real-time PCR should also be carried out to confirm the upregulation of the expression of thiamine biosynthesis genes. Besides that, studies on the other genes encoding for other enzymes in the thiamine biosynthesis pathway in oil palm will be useful in elucidating the overall regulation of thiamine biosynthesis in oil palm. It is believed that overexpression of thiamine or the specific enzymes in thiamine biosynthesis pathway will contribute to a more stress-tolerant oil palm variety.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was carried out in Lab 230, Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia with the financial support of “Geran Putra Universiti Putra Malaysia (Project No.: 9425900)”, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

REFERENCES

Ache P, Becker D, Ivashikina N, Dietrich P, Roelfsema MRG, Hedrich R (2000). GORK, a delayed outward rectifier expressed in guard cells of *Arabidopsis thaliana*, is a K+-selective, K+-sensing ion channel; Febs Letters 486:93-98.

Ahn IP, Kim S, Lee YH (2005). Vitamin B_1 functions as an activator of plant disease resistance; Plant Physiol. 138:1505-1515.

Balia Yusof ZN, Borhan FP, Mohamad FA, Rusli MH (2015). The effect of *Ganoderma boninense* infection on the expressions of thiamine (vitamin B_1) biosynthesis genes in oil palm; J. Oil Palm Res. 27:12-18.

Chatterjee A, Schroeder FC, Jurgenson CT, Ealick SE, Begley TP (2008). Biosynthesis of the thiamin-thiazole in eukaryotes: Identification of a thiazole tautomer intermediate; J. Am. Chem. Soc.130:11394-11398.

Frank RAW, Leeper FJ, Luisi BF (2007). Structure, mechanism and catalytic duality of thiamine-dependent enzymes; Cellular and Molecular Life Sciences, 64:892-905.

Gill SS and Tuteza N (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants; Plant Physiol. Biochem. 48:909-930

Goyer A (2010). Thiamine in plants: Aspects of its metabolism and functions. Phytochemistry 71:1615-1624.

Hoffmann S (2002). Osmotic stress signaling and osmoadaptation in yeasts. Microbiol. Mol. Biol. Rev. 66:300-372.

Hosy E, Vavasseur A, Mouline K, Dreyer I, Gaymard F, Porée F, Boucherez J, Lebaudy A, Bouchez D, Véry A A and Simonneau T (2003). The *Arabidopsis* outward K+ channel GORK is involved in regulation of stomatal movements and plant transpiration; Proc. Natl. Acad. Sci. 100:5549-5554.

Kim EJ, Kwak JM, Uozumi N, Schroeder JI (1998). AtKUP1: an *Arabidopsis* gene encoding high-affinity potassium transport activity; Plant Cell 10:51-62.

Lebaudy A, Véry A A and Sentenac H (2007). K+ channel activity in plants: genes, regulations and functions; FEBS Letters 581:2357-2366.

Li Z, Trick HN (2005). Rapid method for high-quality RNA isolation from seed endosperm containing high levels of starch; BioTechniques 38:872-876.

Machado CR, de Oliveira RLC, Boiteux S, Praekelt UM, Meacock PA Menck CFM (1996). Thi1, a thiamine biosynthetic gene in *Arabidopsis thaliana*, complements bacterial defects in DNA repair; Plant Mol. Biol. 31:585-593.

Machado CR, Praekelt UM, de Oliveira RC, Barbosa ACC, Byrne KL, Meacock PA, Menck CFM (1997). Dual role for the yeast THI4 gene in thiamine biosynthesis and DNA damage tolerance; J. Mol. Biol. 273:114-121.

Maggio A, Zhu J K, Hasegawa P M and Bressan RA (2006). Osmogenetics: aristotle to *Arabidopsis*; Plant Cell 18:1542-1557.

Moulin M, Nguyen GTDT, Scaife MA, Smith AG, Fitzpatrick TB (2013). Analysis of *Chlamydomonas* thiamin metabolism in vivo reveals riboswitch plasticity; Proc. National Acad. Sci. United States Am. 110:14622-14627.

Munns R, Passioura JB, Guo J, Chazen O, Cramer GR (2000). Water relations and leaf expansion: Importance of time scale. J. Exp. Bot. 51:1495-1504.

Nosaka K (2006). Recent progress in understanding thiamin biosynthesis and its genetic regulation in *Saccharomyces cerevisiae*; Appl. Microbiol. Biotechnol. 72:30-40.

Osakabe Y, Arinaga N, Umezawa T, Katsura S, Nagamachi K, Tanaka H, Ohiraki H, Yamada K, Seo SU, Abo M, Yoshimura E (2013). Osmotic stress responses and plant growth controlled by potassium transporters in *Arabidopsis*; Plant Cell, 25:609-624.

Osakabe Y, Kajita S, Osakabe K (2011). Genetic engineering of woody plants: current and future targets in a stressful environment; Physiol. Plant. 142:105-117.

Pourcel L, Moulin M, Fitzpatrick TB (2013). Examining strategies to facilitate vitamin B_1 biofortification of plants by genetic engineering; Front. Plant Sci. 4:1-8.

Rapala-Kozik M, Kowalska E and Ostrowska K (2008). Conditions of abiotic stress; J. Exp. Bot. 59:4133-4143.

Rapala-Kozik M, Wolak N, Kujda M, Banas AK (2012). The upregulation of thiamine (vitamin B 1) biosynthesis in *Arabidopsis thaliana* seedlings under salt and osmotic stress conditions is mediated by abscisic acid at the early stages of this stress response. BioMed Central Plant Biol. 12(1):1.

Raschke M, Bürkle L, Müller N, Nunes-Nesi A, Fernie AR, Arigoni D Fitzpatrick TB (2007). Vitamin B_1 biosynthesis in plants requires the essential iron-sulfur cluster protein, ThiC; Proc. Natl. Acad. Sci. United States Am. 104:19637-19642.

Ribeiro DT, Farias LP, De Almeida JD, Kashiwabara PM, Ribeiro AF, Silva-Filho M C, Menck C F M and Van Sluys M A (2005) Functional characterization of the thi1 promoter region from *Arabidopsis thaliana*. J. Exp. Bot. 56:1797-1804.

Pei ZM, Kuchitsu K, Ward JM, Schwarz M, Schroeder JI (1997). Differential abscisic acid regulation of guard cell slow anion channels in *Arabidopsis* wild-type and abi1 and abi2 mutants. Plant Cell 9:409-423.

Schroeder JI, Hagiwara S (1989). Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells; Nature 338:427-430.

Tunc-Ozdemir M, Miller G, Song L, Kim J, Sodek A, Koussevitzky S,Shintani D (2009). Thiamin confers enhanced tolerance to oxidative stress in *Arabidopsis.* Plant Physiol. 151:421-432.

Upadhyaya H, Panda SK (2004). Responses of *Camellia sinensis* to drought and rehydration; Biol. Plant 48:597-600.

Upadhyaya H, Panda SK, Dutta BK (2008). Variation of physiological and antioxidative responses in tea cultivars subjected to elevated

water stress followed by rehydration recovery; Acta Physiol. Plant 30:457-468.
Upadhyaya H, Panda SK, Dutta BK (2011). CaCl2 improves post drought recovery potential in *Camellia sinensis* (L) O. Kuntze; Plant Cell Rep. 30:495-503.
Upadhyaya H, Sahoo L, Panda SK (2013). Molecular physiology of osmotic stress in plants. Mol. Stress Physiol. Plants 7:179-192.
Zhang W, Han DY, Dick WA, Davis KR Hoitink HAJ (1998). Compost and compost water extract-induced systemic acquired resistance in cucumber and *Arabidopsis*. Phytopathology 88:450-455.
Zonia L, Munnik T (2007). Life under pressure: hydrostatic pressure in cell growth and function; Trends Plant Sci. 12:90-97.

academicJournals

Vol. 15(29), pp. 1575-1596, 20 July, 2016
DOI: 10.5897/AJB2016.15310
Article Number: 737E4BF59637
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Identification and analysis of cassava genotype TME3 bacteria artificial chromosome libraries for characterization of the cassava mosaic disease

Paul Kuria[1,2,3]*, Elijah Ateka[1], Douglas Miano[4], Justus Onguso[1] and Nigel J. Taylor[3]

[1]Jomo Kenyatta University of Agriculture and Technology, P. O. Box 62000-00200, Nairobi, Kenya.
[2]Kenya Agricultural and Livestock Research Organization, P. O. Box 57811-00200, Nairobi, Kenya.
[3]Donald Danforth Plant Science Center, 975 North Warson Road, St Louis, MO, 63132 USA.
[4]University of Nairobi, P. O. Box 30197, 00100, Nairobi, Kenya.

Received 26 February, 2016; Accepted 10 June, 2016

Cassava is an economically important crop in sub-Saharan Africa; however, its yield potential is constrained by cassava mosaic disease (CMD) infection. Classical genetics and biotechnology are being harnessed to overcome the disease and secure yields for farmers. The CMD2 resistance locus flanked by three simple sequence repeats (SSR) markers and one sequence characterized amplified region (SCAR) marker were mapped in West African genotypes and shown to impart qualitative resistant to all species of CMGs. However, gene(s) associated with the CMD2 locus and their mode of actions remains unknown. In an effort to discover gene(s) located in CMD2 locus region, TME3 BAC collections were screened for the presence of CMD2 flanking markers. CMD susceptible and resistant cassava genotypes were found to contain 100% of the markers flanking CMD2 locus. SNPs and nucleotide deletions were identified within the marker sequences but there was no evidence of trait and marker association. All the SSR markers flanking CMD2, and the more recently characterized CMD3 loci were to be located on chromosome 12. Through BAC pools library hybridization with marker probes, 130 BACs were identified, but only 23 BACs contained at least CMD2 specific two markers. Whole BAC sequencing identified five clones that mapped to the marker regions. BAC29 assembled into a 100 kb contig and encoded tandem repeats of three full length R genes (3.5 kb) and two partial repeats. These R genes were conserved and highly expressed in CMD susceptible and CMD resistant cassava genotypes. Promoter sequences derived from R genes showed similar transient expression of GUS as 35S promoter. On cassava genome V6.1 BAC29 sequences were mapped to chromosome 16, eliminating their potential role in CMD resistant.

Key words: Bacteria artificial chromosome, CMD2, cassava, cassava mosaic disease.

INTRODUCTION

Cassava genetic improvement through classical breeding and biotechnology targets various traits such as disease and pest resistance, enhanced nutritional and reduced cyanogenic content, improved starch quality as well as

decreased post-harvest deterioration (Legg et al., 2015; Akinbo et al., 2012; Welsch et al., 2010; Jørgensen et al., 2005; Lokko et al., 2005; Siritunga et al., 2004). In sub-Saharan Africa, cassava production is constrained by two viral diseases namely Cassava mosaic disease (CMD) caused by multiple species of single stranded DNA viruses in the family geminiviridae, and Cassava brown streak disease (CBSD) caused by two species of RNA viruses in the family Potyviridae (Legg et al., 2011, 2015). CMD is endemic to all cassava production regions of sub-Saharan Africa (Harimalala et al., 2015; Legg et al., 2015; Patil and Fauquet, 2009). As a result, classical breeding programs have focused significant attention on mitigating the negative effects caused by CMD. Through marker assisted breeding, three quantitative trait loci (QTL) namely CMD1, CMD2 and CMD3 have been independently mapped in cassava genotypes namely TMS30572, TME3 and TMS97/2205 (Rabbi et al., 2014; Okogbenin et al., 2012; Lokko et al., 2005; Akano et al., 2002; Fregene and Puonti-Kaerlas 2002). During the 1980s and 1990s, a CMD epidemic occurred in East Africa. A successful approach in managing the crisis was achieved through deployment of the West African landraces TME14 and TME204 which carry the CMD2 locus (Legg, 1999). These cultivars are now among the most popular genotypes in Kenya and Uganda due to their inherent resistance to CMD, good cropping characteristics, cooking qualities and taste. However, they are highly susceptible to CBSD. Others traditional East African cultivars, such as Ebwanatereka, possess desirable organoleptic traits but are highly susceptible to both CMD and CBSD (Kaweesi et al., 2014). Transgenic technologies allow the introduction of multiple traits into an individual cultivar (Taylor et al., 2012). Various breeding programs and research groups are embarking on the use of such techniques to stack multiple resistance traits onto genetic backgrounds with inherent resistance to CMD and improving the popular but CMD susceptible genotypes (Chauhan et al., 2015).

The West African cassava landraces carrying the CMD2 resistant locus are popular targets for genetic engineering (Beyene et al., 2015; Vanderschuren et al., 2012; Taylor et al., 2012) and as parents for cassava genetic improvement programs (Rabbi et al., 2014; Okogbenin et al., 2012; Akano et al., 2002). Qualitative CMD resistance conferred by CMD2 is reported to be stably inherited as a single locus and to confer broad specificity to cassava-infecting geminiviruses (Rabbi et al., 2014; Okogbenin et al., 2007). CMD2 has been mapped on the genetically similar cassava landraces TME1, TME7 and TME14 (Rabbi et al., 2014) using molecular markers designated as sequence characterized amplified region (SCAR) marker RME1, simple sequence repeats (SSR) markers; SSRY28, NS158 and NS169 and single nucleotide polymorphism (SNP) markers (Rabbi et al., 2014; Lokko et al., 2005; Akano et al., 2002). SSR and SNP markers linked with the CMD2 locus are collocated on chromosome 12 of cassava genome version 6.1 (International Cassava Genetic Map Consortium [ICGMC] 2015). The current version of cassava genome anchor 72% (382 MB) of cassava sequences on genetic map (ICGMC, 2015) and genomic scan identified gaps within the chromosomal region carrying CMD2 locus. This indicates that there are a significant number of genes missing from the existing cassava reference genome, especially within highly repetitive regions. Therefore, in its current form, the cassava reference genome is not sufficiently reliable to unravel all sequences coding for genes harbored in CMD2 locus. In addition, the sequenced cassava genome was derived from the partial inbred line AM560-2, a progeny of the Latin-American cassava cultivar MCOL1505 (Prochnik et al., 2012).

According to Okogbenin et al. (2007), all cassava germplasm originating from South America are susceptible to CMD. This means that alleles specific to CMD resistance are most likely not present in the AM560-2 genome. In the absence of a fully sequenced and annotated cassava genome from a CMD resistant genotype, bacterial artificial chromosomes (BACs) were adopted to construct large insert libraries for positional cloning of the CMD2 locus in cassava landrace TME3. This covered 10.1X of the haploid genome, indicating 99% chance of finding any given sequence (Tomkins et al., 2004). BACs are preferred as they enabled stable cloning of large genome insert sizes of 100-200 kb and can be propagated easily in *E. coli* cells without undergoing rearrangements (Zhang et al., 2012; Shi et al., 2011). In genomics, large-insert DNA libraries are utilized in the development of physical maps of the genome (Tomkins et al., 2004), for high throughput sequencing of genomes (Prochnik et al., 2012), and in map-based cloning of agronomically important genes such as those imparting disease resistance (Ragupathy et al., 2011). Using BAC by BAC sequencing, resistance gene analogues (RGAs) were isolated and mapped from TMS30001, a CMD resistant genotype derived from *Manihot glaziovii,* and from cultivar MECU72 carrying whitefly resistant loci (Tomkins et al., 2004). Recently, high quality draft-genomes have been assembled through integration of BAC-based physical maps and BAC-end sequences (Wang et al., 2014; International Barley Genome Sequencing Consortium, 2012), indicating the power of the resources and associated technologies for elucidating genomic information in crop plants.

Map based cloning of cassava was initiated through

*Corresponding author. E-mail: kuriapk@gmail.com. Tel: +254736333294.

collaborative efforts by the International Center for Tropical Agriculture (CIAT) and Clemson University Genomics Institute (CUGI) in an effort to identify genes coding for important traits. Through this project, TME3 BAC libraries were constructed and markers in the vicinity of CMD2 locus identified (CIAT, 2006). BAC end sequencing of clones carrying markers BAC33a, BAC33b, BAC36b and SBAC33c were constructed into 13 contigs spanning across the CMD2 region based on the position of markers RME1 and BAC33b located on either side of the CMD2 locus (CIAT, 2007).

In the present study, attempts were made to assemble sequences and identify genes residing in the CMD2 locus. Thirteen BAC clones were obtained from CIAT courtesy of Dr Luis Augusto, and additional BACs libraries hybridizing to the CMD2 flanking markers were procured from CUGI. Results described here reported presence of CMD2 and CMD3 loci flanking markers in all cassava genotypes irrespective of CMD resistant status. TME3 BAC library hybridization with markers flanking CMD2 locus and whole BAC sequencing identified sequences mapping to markers on either side of CMD2. However, contigs were not long enough to transverse the entire CMD2 locus region. Tandem repeats of five disease resistance genes were identified in one of the clones and their diversity studied in different cassava genotypes. However, they were mapped to chromosome 16 of the current cassava reference genome.

MATERIALS AND METHODS

Selection of candidate BAC libraries spanning around CMD2 locus

Four markers that mapped closest to the CMD2 locus and described by Okogbenin et al. (2012) were PCR amplified from cassava genotype TME3 using the conditions and primer sequences reported by Okogbenin et al. (2012). Amplicons corresponding to the full length marker DNA sequence were cloned into Zero blunt Topo vector (Life Technologies, Carlsbad, CA, USA) and confirmed through sequencing using M13-F (5´-GTAAAACGACGGCCAG-3') and M13-R (5´-CAGGAAACAGCTATGAC-3´) primers.

Four high density colony filters were hybridized with each probe to cover the entire TME3 BAC libraries. Hybridizations were performed overnight at 60°C, followed by two stringent washes at 60°C with 0.1% SDS and 1X SSC for 1 h. Images of the hybridizations were recorded by phosphor screens and read by a Typhoon 9400 imager (GE Healthcare, Piscataway, NJ, USA). The coordinates of the BAC clones were identified on filters using Hybdecon software (CUGI). Bacterial clones corresponding with positive signals were isolated using a sterile toothpick and grown overnight in 3 ml of liquid LB medium containing 12.5 µg/µl chloramphenicol. Plasmid DNA was extracted from three colonies per BAC using PureLink® Quick Plasmid Miniprep Kit (Life Technologies, Carlsbad, CA, USA). The BAC hybridization results were verified through PCR using CMD2 flanking marker primers as reported by Okogbenin et al. (2012) and described in Supplementary Table 1. Based on PCR results, ten BACs were selected and submitted to Dow Agro Science (Indianapolis, IN, USA) for whole insert sequencing. An additional thirteen BAC clone libraries designated, BAC12, BAC16, BAC21, BAC22, BAC23, BAC26, BAC29, BAC33, BAC31, BAC38, BAC40, BAC44 and BAC45 reported to have been constructed around the CMD2 locus (CIAT, 2007) were procured from CIAT and submitted to the Genome Technology Access Center at Washington University in St Louis for paired end MiSeq sequencing.

BAC sequence assembly and annotation

Raw sequences obtained from full BAC sequencing were downloaded, demultiplexed by QIIME (Caporaso et al., 2010) and trimmed using Cutadapt (Martin, 2011) to remove adaptor sequences followed by filtering of sequences of bacteria and vector origin by randomized Numerical Aligner (Vezzi et al., 2012) Clean reads from each BAC were assembled using Sanger Sequence Assembly Software (DNASTAR) using default settings and all contigs larger than 5 kb used for the present analysis. Comparative analysis of BAC sequences was then performed to identify cassava genomic regions harboring homologous sequences.

Coding regions of BAC sequences were identified through BLASTN using cassava EST sequences (http://cassava.igs.umaryland.edu/blast/db/EST_asmbl_and_single.fasta) as query and the BAC clone sequences as the subjects. The potential coding sequences were blasted against cassava genome V6.1 (http://phytozome.jgi.doe.gov/) and homologous genes identified based on functional annotation. To increase the level of confidence, homology searches in NCBI were performed to reveal putative genes based on 95-100% nucleotide identities. For disease resistant genes, conserved protein domains were identified using HMMER software suite (http://pfam.xfam.org/). Primers described in Supplementary Table 1 were used to study diversity of full length disease resistant genes in various cassava genotypes.

Amplification of putative CMD resistance genes from cassava genotypes

From one of the BACs sequenced, hereafter referred to as BAC29, three tandem repeats of putative full length resistance (R) genes containing motifs for coiled-coil nucleotide binding site leucine rich repeat (CC-NBS-LRR) and two truncated R genes were identified. To identify nucleotide diversity of the R genes in cassava genotypes, primers were designed from BAC29 sequences to independently amplify 3.5 and 1.6 kb of full length and truncated R genes, respectively (Supplementary Table 1). The promoter regions of putative CMD2 gene(s) was targeted for amplification using primers designed upstream of the genes to cover 1.5-2.0 kb (Table 1). Total DNA was extracted from CMD2 types cassava genotypes TME3, Oko iyawo, TME7, TME204, CMD1 types TMS30001, TMS30572, CMD3 types TMS97/2205 TMS98/0505 and susceptible types TME117, 60444 and Ebwanatereka, using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was quantified on NanoDrop (Thermo scientific Waltham, MA, USA) and 100 ng mixed with 22 µl AccuPrime™ Pfx SuperMix (Life Technologies, Carlsbad, CA, USA), 1 µl of each primer (0.5 µM final concentration) and subjected to the following PCR conditions; one cycle of 5 min at 94°C followed by 35 cycles of amplification (35 s at 94°C, 30 s at 52°C and 3 min (R gene ORF), 1.5 min (promoter) at 68°C) and a final cycle of 10 min at 68°C. The PCR amplicons were analyzed by running on a 1% (w/v) agarose gel electrophoresis at 120 V for 30 min. The desired fragments were excised from the gel and purified using PureLink® Quick Gel Extraction Kit (Life Technologies, Carlsbad, CA, USA) as per the manufacturer's instructions.

Cloning putative R genes and their endogenous promoters

The gel purified genes and promoter PCR products were cloned

Table 1. Markers flanking CMD resistant loci. All the markers associated with CMD resistance were located in different genomic regions of chromosome12.

Marker name	Chromosomal location V6.1	Length	Cassava gene carrying marker
SSRY28	Chromosome12:7541033..7541198	166 bp	Manes.12G074000 (5th exon and 4th intron)
SSR NS158	Chromosome12:7731640..7731816	177 bp	Manes.12G074900 (3rd intron)
SSR NS169	Chromosome12:7731640..7731970	331 bp	Manes.12G074900 (3rd intron and 4th exon)
RME1	Scaffold01154:5660..6055 Scaffold01154:14884..15045 Scaffold01154:12726..12851	740 bp	Multiple R genes
NS198	Chromosome12:1353173..1353364	192 bp	Manes.12G016800 (2nd intron)
GBS-SNP	Chromosome12:7216154..7223867		Manes.12G071900
GBS-SNP	Chromosome12:6910006..6917228		Manes.12G069800
GBS-SNP	Chromosome12:6640962..6649139		Manes.12G068200

into Zero Blunt TOPO cloning vector following the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA). Two microliters of ligation mix was transformed into 50 µl of One Shot TOP10 chemically competent *E. coli* cells (Life Technologies, Carlsbad, CA, USA) as described by the manufacturer. After one hour incubation at 37°C in 250 µl LB medium minus antibiotics, 50 µl of transformed cells were plated on LB agar plates containing 50 µg/ml kanamycin, 100 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 20 mg/ml X-gal and incubated overnight at 37°C. Six individual white colonies per clone were grown in liquid LB media containing 50 µg/ml kanamycin for 8 h. Plasmid DNA was purified using Purelink Quick plasmid miniprep kit (Life Technologies, Carlsbad, CA, USA) and confirmed for insert size through double digestion of 100 ng plasmid DNA with *Xma*I and *Xba*I restriction enzymes (New England Biolabs, Ipswich, MA USA). Six clones per cassava genotype were submitted to Genewiz (http://www.genewiz.com) for Sanger sequencing using M13-F, M13-R, and additional primers designed in the middle of gene to cover the entire open reading frame (ORF), shown in Supplementary Table 1. The sequence contigs were assembled using SeqMan Pro and aligned with MegAlign Pro of DNASTAR Lasergene 12.2 (DNASTAR, USA). Maximum likelihood phylogenetic trees were constructed to study the diversity of sequences. The sequences of putative promoters were analyzed for promoter elements using program PLACE web Signal Scan (http://www.dna.affrc.go.jp/PLACE/signalup.html). To study the promoter activities, clones were constructed into binary vector pCambia5000 using promoter sequences derived from CMD resistant and susceptible genotypes respectively to drive the expression of beta-glucuronidase (GUS) reporter gene whereas 35s promoter was used as the control. The promoter activity was determined through leaf infiltration transient expression in *Nicotiana benthamiana* following the procedures described by Wroblewski et al. (2005).

Northern blot analysis of potential R genes

To determine the level of putative R gene expression in different cassava genotypes, total RNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle 1990). Following extraction, RNA was incubated at 37°C for one hour with 4 µl of TURBO DNA-free™ Kit (Life Technologies, Carlsbad, CA, USA) to remove contaminating DNA. The RNA was quantified on NanoDrop (Thermo scientific Waltham, MA, USA) and 10 µg electrophoresed in 1% denaturing agarose gel for 2 h at 80 V before transfer to a positively charged Hybond nylon membrane (Amersham, UK) using DEPC treated 20X SSC (0.3 M trisodium citrate and 3.0 M sodium chloride) for 12 h. Membrane bound RNA was subjected to UV at 120,000 microjoules/cm^2 using a Stratalinker UV crosslinker 1800 (Stratagene, La Jolla, CA) and pre hybridized in Digoxigenin (DIG) Easy Hyb solution (Roche, Indianapolis, IN, USA) for 1 h and hybridization overnight at 42°C with DIG-labeled probes specific to each R gene.

Cloning of TOM1 gene homolog from cassava

A protein homologous to tobamovirus multiplication protein 1 (TOM1) designated Manes.16G009700.1 was identified in the same genomic region harboring a cluster of NBS-LRR genes that were homologous to BAC29 R genes. Primers TOM1-1 AGAGAATGACCAGAATGCCAGTGC and TOM1-2 TTACCGAATAGGGTGATATTGCGCCG were used to amplify 850 bp transcript of the gene from cassava genotypes TME3, TME204, 60444 and Ebwanatereka. The RT-PCR products were cloned into Zero Blunt TOPO vector and transformed into one shot TOPO ten competent cells. Colonies were screened and positive transformants were sequenced. Sequence contigs were assembled using SeqMan Pro and aligned with MegAlign Pro of DNASTAR Lasergene 12.2 (DNASTAR, USA).

RESULTS

Analysis of CMD2 locus flanking markers in different cassava genotypes

Cassava genotypes possessing resistance or susceptibility to CMD were evaluated for the presence of markers flanking CMD2 and CMD3 resistance loci. Analysis of CMD2 flanking markers revealed presence of two RME1 marker alleles with the upper band corresponding to 740 bp while the shorter fragment was about 500 bp in size (Figure 1A). Two alleles of RME1 were identified in cassava genotypes TME117, Ebwanatereka (both CMD susceptible) and in

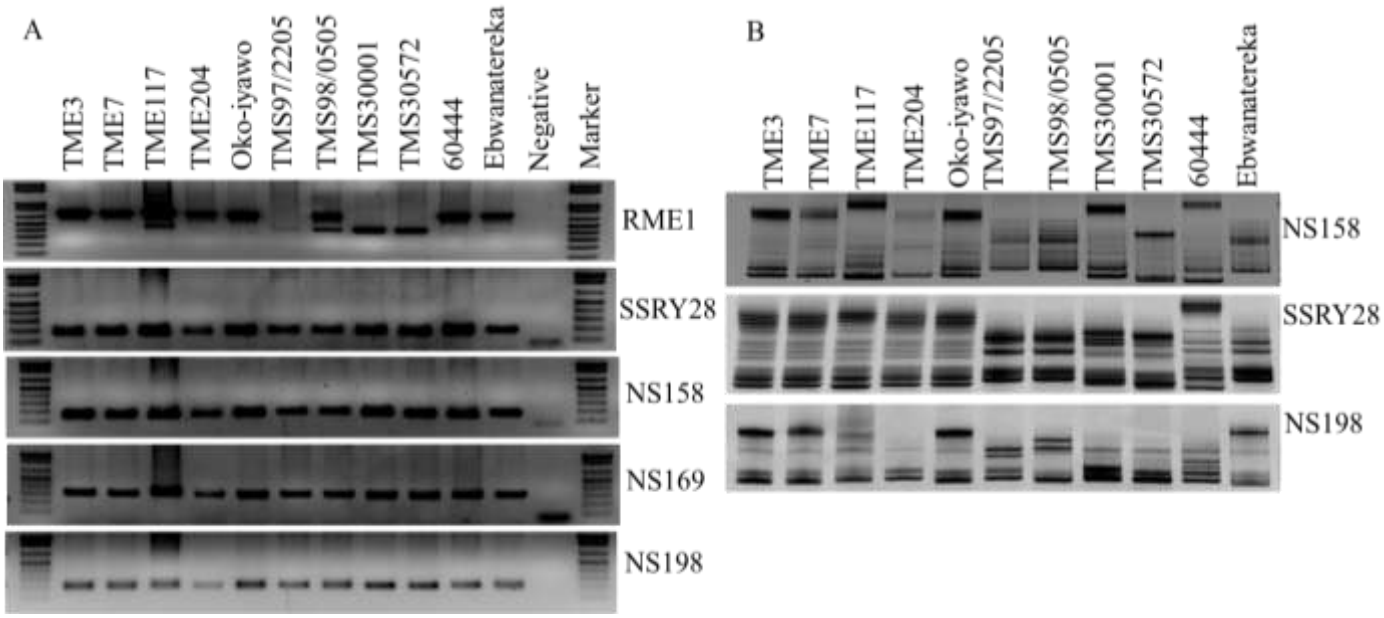


Figure 1. Analysis of cassava genotypes for the presence of markers flanking CMD resistance locus. (A) The PCR products were resolved on 1% (w/v) agarose gel for 1 h at 100 V. (B) Single-strand conformation polymorphism of SSR markers analyzed on 8% non-denaturation polyacrylamide gel electrophoresis.

TMS98/0505 (CMD resistant). One allele of RME1 corresponding to 740 nt was observed in cassava genotypes TME3, TME204, Oko-iyawo, (all CMD resistant), TME7 and 60444 (both CMD susceptible). On the other hand, genotypes conferring quantitative CMD resistance mediated by CMD1 (TMS30572) and CMD3 (TMS97/2205) respectively contained only the shorter, 500 bp sized fragment (Figure 1A). Sequence analysis of the two RME1 alleles revealed that RME1 sequences encode for a partial nucleotide binding motif of a disease resistance gene. Homology match of RME1 sequences on AM560-2 genome revealed imperfect matches, an indication of highly repetitive sequence (Figure 1A).

The SSR markers flanking the CMD2 and CMD3 loci were found to be present in all cassava genotypes (Figures 1A and B). However, analysis of sequences using single stranded conformation polymorphism (SSCP) showed differences across the genotypes (Figure 1B). Sequence analysis of SSRY28 derived from TME3 and 60444 respectively revealed a 12 nucleotide deletion in TME3 starting from position 114. The position of CMD2 flanking markers was identified as follows: SSRY28 on chromosome12:7541033 - 7541198, NS169 on chromosome12:7731640 – 7731970, NS158 on chromosome12:7731640 - 7731816 whereas CMD3 flanking marker NS198 was located on chromosome12:1353345 – 1353192 of cassava genome v6.1 (Table 1). Based on the positions of the markers flanking CMD2 and CMD3 loci, the CMD3 locus is located upstream of the CMD2 locus in the same cassava genomic region.

High-throughput TME3- BAC pools library hybridization with CMD2 marker probes

The four cassava genetic markers RME1, SSRY28, NS169 and NS158 were PCR amplified from cassava genotype TME3, cloned into zero blunt topo and confirmed with *Eco*RI restriction digestion (Figure 2A). Each marker probe was independently hybridized to two replicates of high density colony filters containing 70,000 TME3 BAC clones and images recorded by phosphor screens and read by a Typhoon 9400 imager (Figure 2B). Eighteen BAC clones hybridized with markers NS169 and NS158 respectively while 23 clones were positive with SSRY28 and 89 with RME1 (Table 2). Cross hybridization was detected only for SSRY markers whereby 23 BACs positively hybridized with atleast two marker probes (Supplementary Table 2).

Amplification of CMD2 markers from candidate BAC clones using PCR

The 23 BAC clones identified to carry more than one marker were subjected to PCR analysis using primers specific to each marker (Figure 3). Ten clones were positively identified with RME1, two clones with SSRY28, six clones with NS158 (Figure 3B and C). None of the clones were positive with NS169 (Figure 3D). The two strategies of BAC screening gave conflicting results, whereby some BAC clones that positively hybridized with marker probe were scored as negative using PCR

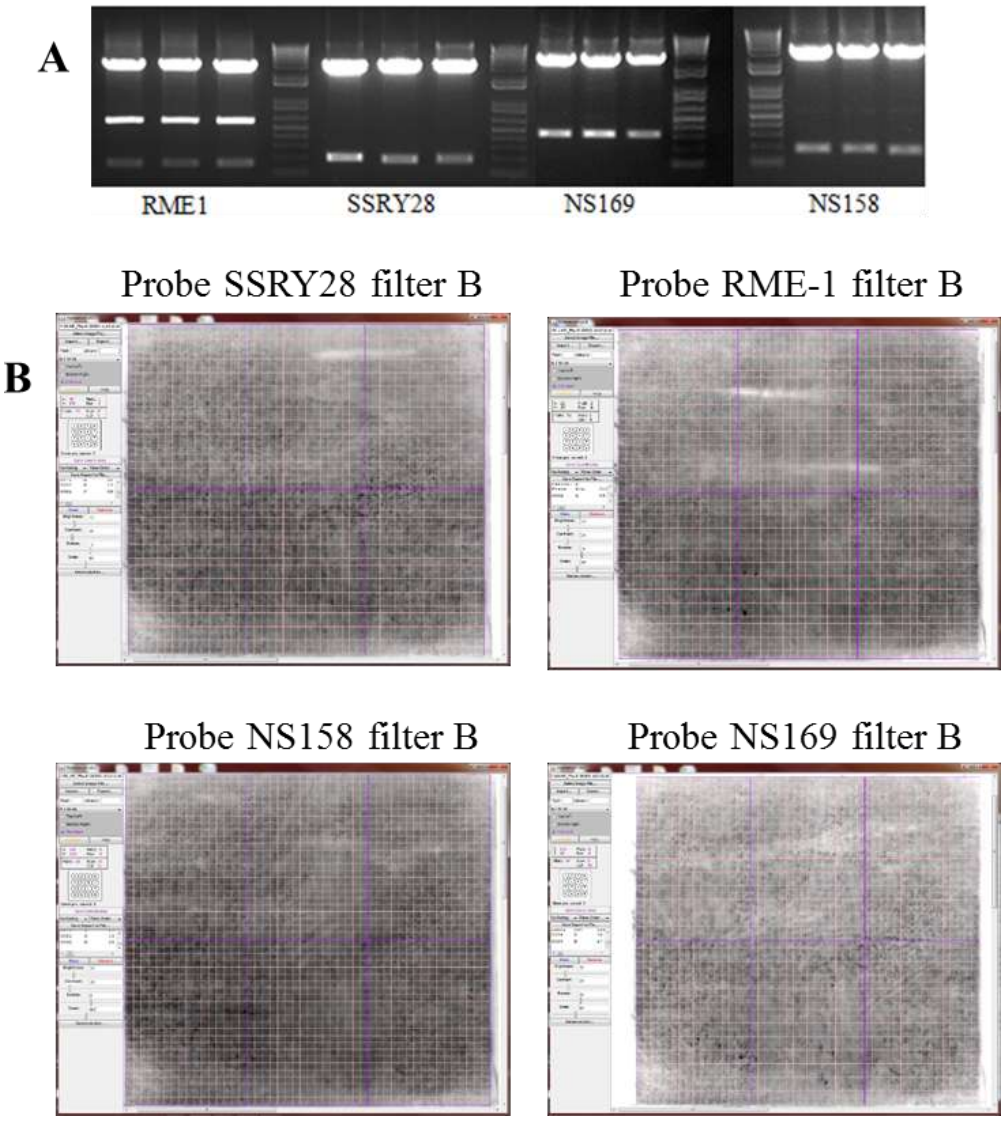


Figure 2. TME3 BAC pool libraries. (A) Restriction digestion of CMD2 locus flanking markers cloned into TOPO vector using EcoRI enzyme. Three independent clones per marker were evaluated. (B) Hybridization of BAC pools libraries on high density filter using with CMD2 marker probes. Filters were deconvoluted and coordinates of positive clones assigned using Hybdecon software.

(Supplementary Table 2). The discrepancy was higher in SSR markers when compared with SCAR marker (RME1). The discrepancy between the two assays may be attributable to contamination of clones during transfer or perhaps non-specific hybridization.

Eleven BAC clones that constructed around BAC33a and SBAC33b as reported by CIAT (2007) were procured from CUGI and further screened with CMD2 mapping markers. PCR analysis of RME1 marker identified a fragment corresponding to 740 bp from five clones (29E13, 70C06, 145G15, 34L16 and 17N21) indicating the presence of RME1 marker (Figure 3E).

Thirteen clones derived from contigs constructed on the bases of mapping marker RME1 and BAC33b (CIAT, 2007) were screened for the presence of CMD2 locus flanking markers by PCR (Supplementary Figure 1). The RME1 marker was detected in BAC23 and BAC32, respectively, while marker NS198 was detected in BAC12 and BAC22 (Supplementary Figure 1 PCR) indicating they were derived upstream of RME1. However, none of the thirteen BACs contained markers NS158, NS169, while SSRY28 mapped downstream of the CMD2 locus (Supplementary Figure 1 PCR). As a result, information pertaining to the position of marker BAC33b or the marker specific primer sequences are not clear and the contigs may therefore have been constructed upstream

Table 2. BAC pool coordinates on high density filters indicating the position of BAC clones that positively hybridized with each of the four markers flanking CMD2 locus.

Markers Flanking CMD2 Locus						
RME1				NS169	NS158	SSRY28
011E08	085P18	110C04	132K11	003H20	003H20	003H20
017N21	086H08	110N24	134H12	012C24	025N10	011E04
020O14	092L05	110O12	136J08	021C10	026L16	021C10
021E05	096M15	114L14	137O22	026N16	026N16	024E16
021O07	096P20	114O03	138A05	033J15	053C09	026L16
022C04	097K13	116A10	144A14	036H04	065H06	026N16
024E14	098B13	116D18	146B02	039D12	070I19	033J15
025E15	098C13	118J05	146G08	042G04	082G13	035I20
025H17	098J09	118P02	146I10	084D15	092N24	079M09
026D16	098J19	119C14	148K05	094B17	099D14	082G13
027O12	099D23	120N03	151C02	106M01	101C17	091J09
027O15	099K04	121G10	154P16	110N21	109O10	100E16
029L10	099M21	122A19	158M04	116N24	110N21	104D06
033P23	099P02	122C19	159C12	126O21	115L19	108L20
049F21	100G16	123L02	162F05	127L07	116N24	109O10
050O10	102B03	125A09	166I15	128N20	120D06	110N21
050P20	102J10	128J08	169G18	146N21	121M18	112N16
051B06	102O06	129B17	171K03	180M18	122F10	116N24
051J24	104E13	130D14	174L05			118F13
064A11	105B12	130D18	184P08			126O21
064F16	105P05	131G02				139N09
069I12	106D17	131O02				159A06
085P12	109B01	132I02				188A02

of the NS158, NS169 and SSRY28 markers.

Mapping of TME3 BAC sequences to the cassava genome

Ten clones identified using hybridization and confirmed using PCR, were fully sequenced (Table 3). Sequences derived from seven BACs met the quality control threshold, allowing further analysis to be performed. The longest contig assembled was 93474 bp in size derived from BAC 52H02, whereas the majority of contigs from other BACs ranged from 5200 to 42000 bp (Table 3). At the chromosomal scale, five of the seven BAC clones were mapped to chromosome XII with two BACs mapping on chromosome II and XVII of cassava genome version 6.1 respectively (Table 3). All five BAC clones identified on chromosome XII were all positive for the presence of the RME1 marker, and based on chromosomal coordinates they were all constructed from the same genomic region (Table 3). However, none of the BACs sequences were homologous to any of the SSR maker sequences (Table 3). Therefore, it was not possible to construct a contiguous sequences spanning from the RME1 marker to SSR markers SSRY28, NS158, and NS169.

Sequencing of BAC clones constructed from the region around CMD2 locus, from markers RME1 marker and BAC33b

Among the thirteen BAC clones previously constructed as reported by CIAT (2007), only one BAC, referred to as BAC29, contained opening reading frames (ORFs) for R genes (Figure 4). This entire BAC was assembled to 100 kb and annotated (Figure 4). Analysis of the BAC29 sequences revealed three tandem repeats of putative R-genes (CMD2a, CMD2b and CMD2c) and two partial R-genes (CMD2d and CMD2e) ORFs in opposite direction (Figure 4). The three NBS-LRR encoding genes identified from TME3 BAC29 were 3.5 kb long with 93-98% nucleotide identity to each other. The truncated R genes were missing two motifs, whereby CMD2d covered only 636 amino acid sequences. While CMD2e was interrupted by a repetitive sequence insertion and

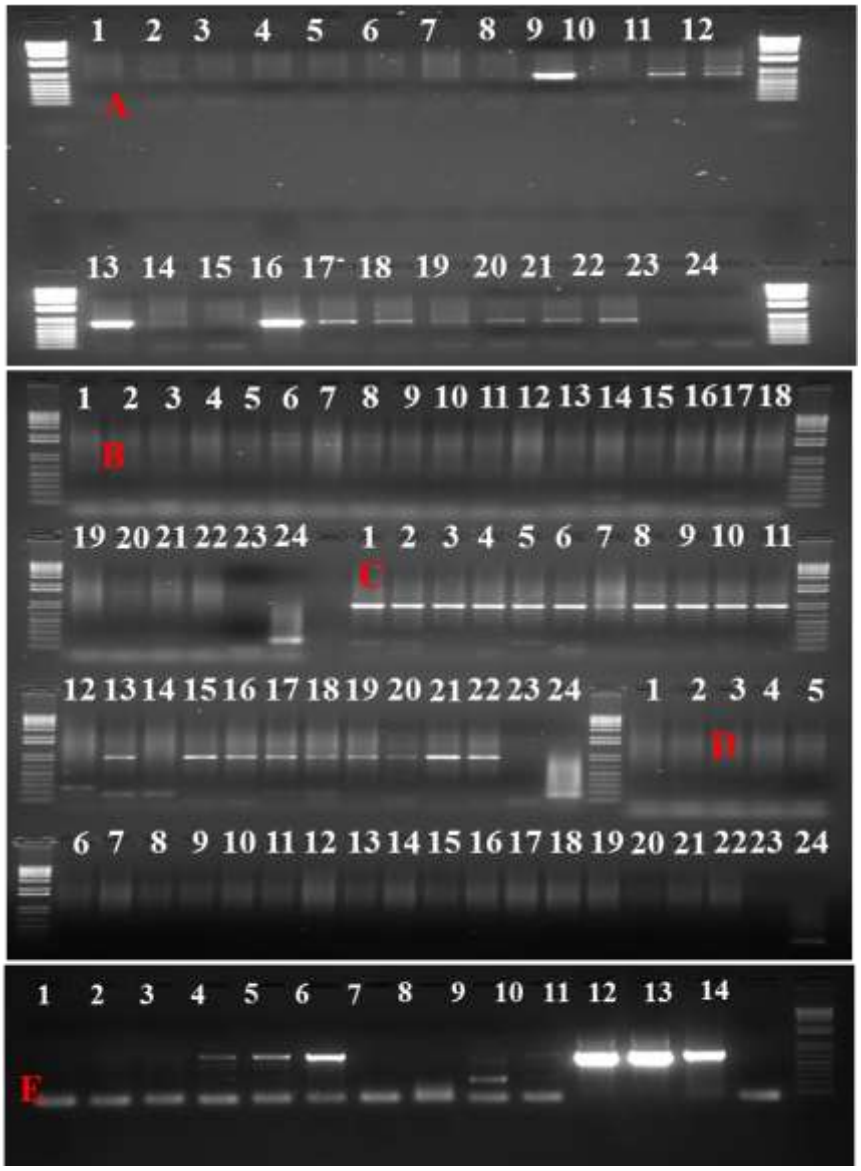


Figure 3. Screening of TME3 BAC clones for the presence of CMD2 flanking marker fragments using PCR. The BACs were screen for the presence of RME1 (A), SSRY28 (B), NS158 (C) and NS169 (D). Numbers denotes each BAC clone as follows: 1: 08J12, 2: 92L05, 3: 98J19, 4: 102O06, 5: 110N21, 6: 109O10, 7: 126O21, 8: 116N24, 9: 17N21, 10: 3H20, 11: 146I10, 12: 159C12, 13: 136J08, 14: 137O22, 15: 22C04, 16: 130D18, 17: 26N16, 18: 21C10, 19: 27O15, 20: 26L16, 21: 33J15, 22: Cassava DNA. (E) BAC clones constructed around RME1 and sBAC33b markers. The clones are identified as follows: 1: 55H06, 2: 53H13, 3: 55H04, 4: 29E13, 5: 70C06, 6: 145G15, 7: 52H02, 8: 55H12, 9: 52H20, 10: 94M08, 11: 34L16, 12: 17N21, 13: Cassava DNA.

contained 30 amino acids deletion at the 3' end (Figure 4).

Sequence analysis of R genes isolated from different cassava genotypes

The full length and truncated R genes were identified in cassava genotypes; TME3, Oko-iyawo, TME7, TME204, TMS30001, TMS30572, TMS97/2205, TMS98/0505, 60444 and Ebwanatereka using PCR and sequencing (Figure 5A). Alignment of contig sequences revealed that the R genes were highly conserved in different cassava genotypes and were clustered into five gene families corresponding to the BAC29 R genes (Figure 5B). However, there were divergent sequences in CMD2A that

Table 3. TME3 BAC clone sequences mapped to AM560-5 genome version 6.1.

BAC	Contig size (bp)	Cassava genomic region	Chromosome Coordinates
130D18	5346 - 34974	Chromosome12	5,604,726 - 5,639,235
136J08	5372 - 35989	Chromosome12	5,697,035 - 5,805,960
145G15	5362 - 40052	Chromosome12	5,609,999 - 5,713,990
52H02	93474	Chromosome2	6,681,446- 6,773,105
52H13	5608 - 39099	Chromosome17	1,014,513-1,079,127
70C06	5362 - 42845	Chromosome12	5,604,775 - 5,663,227
17N21	5346 - 29687	Chromosome12	5,604,726 - 5,699,674

showed 100% nucleotide identities with CMD2B and CMD2C (Figure 5B).

Northern blot analysis of putative CMD2 genes in cassava

Expression of CMD2A, CMD2B and CMD2C in cassava genotypes TME3, Oko iyawo, TME7, TME204, TMS30001, TMS30572, TMS97/2205, TMS98/0505, 60444 and Ebwanatereka was determined from 10 µg of cassava total RNA extracted from leaves of greenhouse grown plants. Based on the intensity of the signal on Northern blot, the expression of CMD2A, CMD2B and CMD2C was uniform across all cassava cultivars regardless of their CMD resistant status (Figure 6). This result indicates CMD2A, CMD2B and CMD2C genes are constitutively upregulated in all cassava genotypes investigated in this study.

Transient expression of GUS driven by putative CMD2 promoter

The ability of the promoter of CMD2A, CMD2B and CMD2C to drive expression of the GUS visual marker gene in *N. benthamiana* was studied. GUS staining was observed tobacco leaves under control of by the putative native CMD2 promoter and 35S promoters (Figure 7) and no difference was observed in the intensity of GUS staining between these two chimeric expression cassettes. GUS was also transiently expressed by putative CMD2 promoters derived from cassava genotypes TME3 and 60444. Based on this result and Northern blot analysis (Figure 6), it can be concluded that the isolated R genes are actively transcribed in different cassava genotypes regardless of their CMD resistant status.

Tobamovirus multiplication protein 1 homolog in cassava

In addition to the disease resistant gene cluster identified (Supplementary Figure 2), a tobamovirus multiplication protein 1 (TOM1) homolog was identified in the same genomic region (chromosome 16:1,005,841- 1,011,074), indicating a locus rich in defense genes. The TOM1 homolog in cassava is encoded by Manes.16G009700 that share 87.5% nucleotide identity with Manes.17G037400 located on chromosome 17:16,718,544 – 16,723,634. Expression of a cassava homolog of TOM1 was identified in genotypes TME3, TME204, 60444 and Ebwanatereka through RT-PCR analysis (Figure 8). The TOM1 family comprises vacuolar membrane proteins that act to inhibit replication of tobamoviruses in tomato when present as mutated alleles (Ishibashi et al., 2010; Yamanaka et al., 2002). Presence of such mutations in CMD resistant or CMD susceptible cassava genotype may be could be implicated in antiviral defense. However, sequence analysis revealed 100% nucleotide identities of TOM1 gene homolog in cassava genotypes TME3, TME204 (both CMD resistant), 60444 and Ebwanatereka (both CMD susceptible) (Figure 8). Based on high sequence similarity of TOM1 homologs in CMD susceptible and resistant cassava genotypes, their involvement in antiviral defense would seem to be unlikely.

DISCUSSION

In this study, molecular markers associated with CMD resistance as reported by Okogbenin et al. (2012) and Rabbi et al. (2014) were identified in all genotypes irrespective of their response to CMD infection. These results agree with Asare et al. (2014) who detected CMD2 flanking markers in both CMD susceptible and resistant genotypes, and contradicts Bi et al. (2010) who reported absence of these markers in the susceptible genotype 60444. Studies by Parkes et al. (2015), reported absence of all marker alleles associated with CMD2 resistance in 18% of F1 progenies derived from a cross between TME11 (CMD2 type) and Dabodabo (local cultivar), despite being resistant to CMD, indicating a different source of CMD resistance. Alternatively, recombination may have occurred in subsequent crossing delinking the marker from the gene, indicating

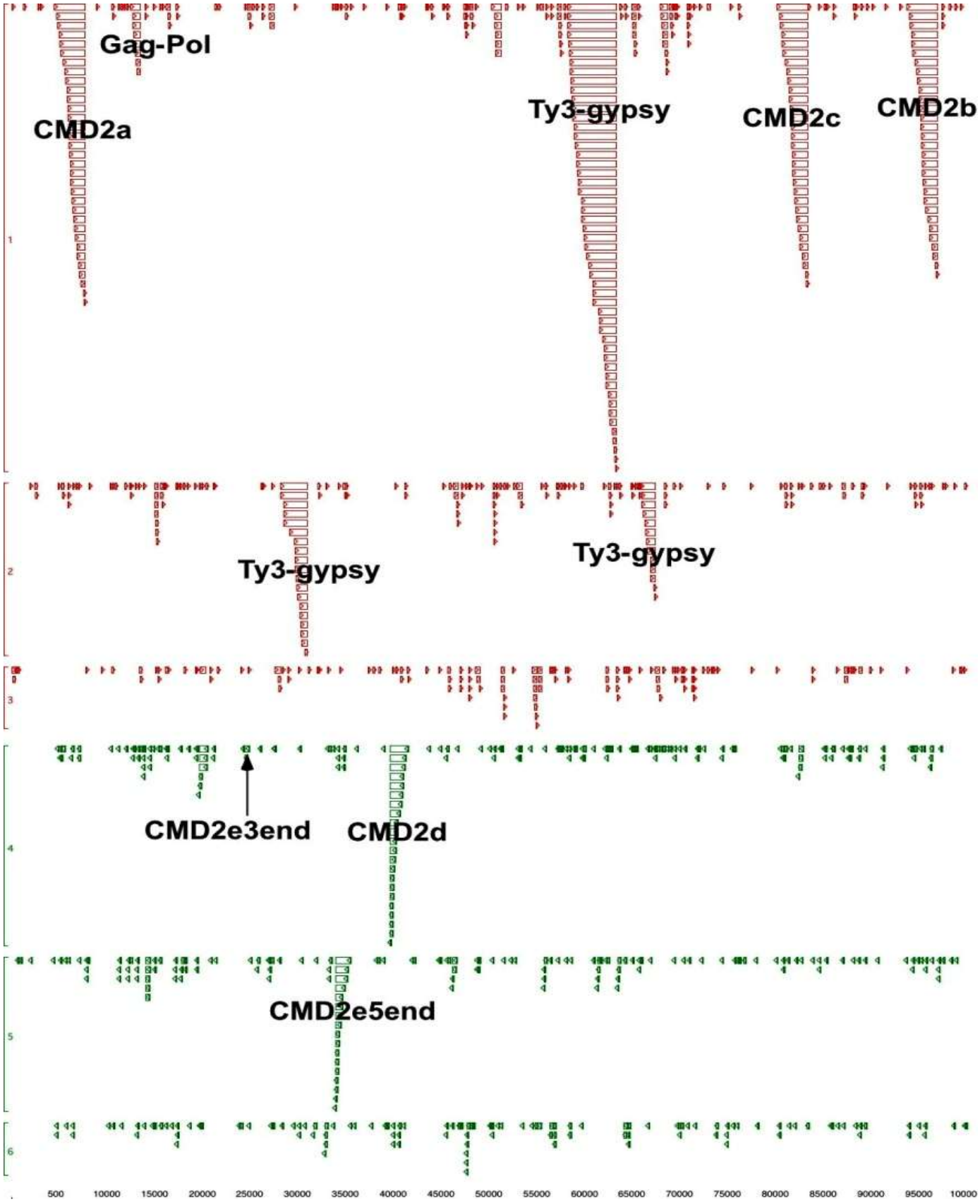
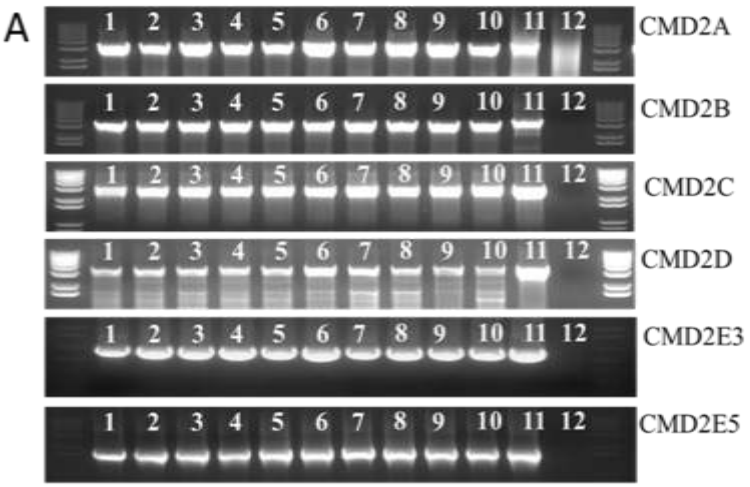


Figure 4. Assembly and annotation of BAC29 derived from TME3 cassava genotype. Several features were identified as follows: CMD2a to e encode putative R genes whereas Gag-pol and Ty3-gypsy represents transposons.



Sample #	Genotype	CMD Status
1	Tme3	Moderate Resistant
2	Tme7	Highly Resistant
3	Tme204	Moderate Resistant
4	Oko-Iyawo	Moderate Resistant
5	TMS97/2205	Highly Resistant
6	TMS98/0505	Highly Resistant
7	TMS30001	Highly Resistant
8	TMS30572	Highly Resistant
9	60444	Susceptible
10	Ebwanatereka	Susceptible
11	BAC29 DNA	Susceptible
12	Water control	Susceptible

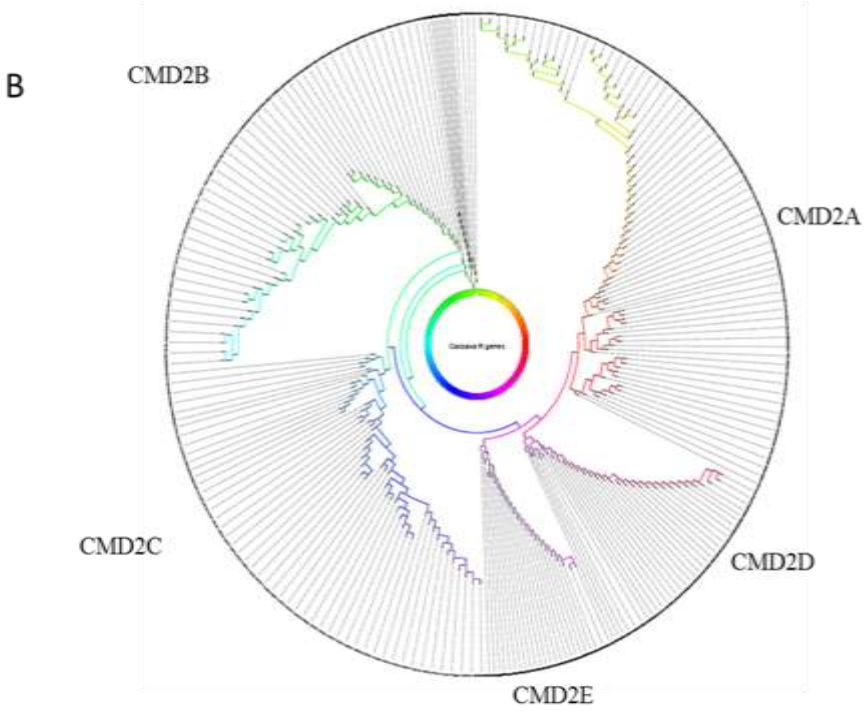


Figure 5. Analysis of R genes R genes from diverse cassava genotypes based on BAC29 sequences. (A) PCR amplification of full length R genes 1: TME3, 2: TME7, 3: TME204, 4: Oko-Iyawo, 5: TMS97/2205, 6: TMS98/0505, 7: TMS30001, 8: TMS30572, 9: 60444, 10: Ebwanatereka, 11: BAC29, 12: Water control. (B) Phylogenetic analysis of putative CMD2 genes from different cassava genotypes. Full length nucleotide sequences were analyzed using aligned MegAlign Pro of DNASTAR Lasergene 12.2.

poor linkage between the markers and the CMD2 resistance mechanism. In cassava genotype TMS97/2205, it was shown here that the RME1 marker was not detected (Figure 1A) as previously reported by Okogbenin et al. (2012). TMS97/2205 has a pedigree of TME6 (CMD2) and TMS30572 (CMD1) (Okogbenin et al., 2012; Dixon et al., 2010) and shows very high resistant to all CMGs under field (Okogbenin et al., 2012) and greenhouse conditions (unpublished data). It is therefore important to stress that data generated in the present study strongly suggests that the CMD2 markers are not close enough to the gene to be reliably used as a fully diagnostic tools for the presence of functional CMD2 resistance. Based on this result, the presence or absence of the previously described marker alleles may not give conclusive evidence of the presence of gene(s) conferring functional resistance to CMD and, importantly, therefore not completely reliable for screening materials during early stages of breeding programs.

The SCAR marker (RME1) and SSR markers (NS158

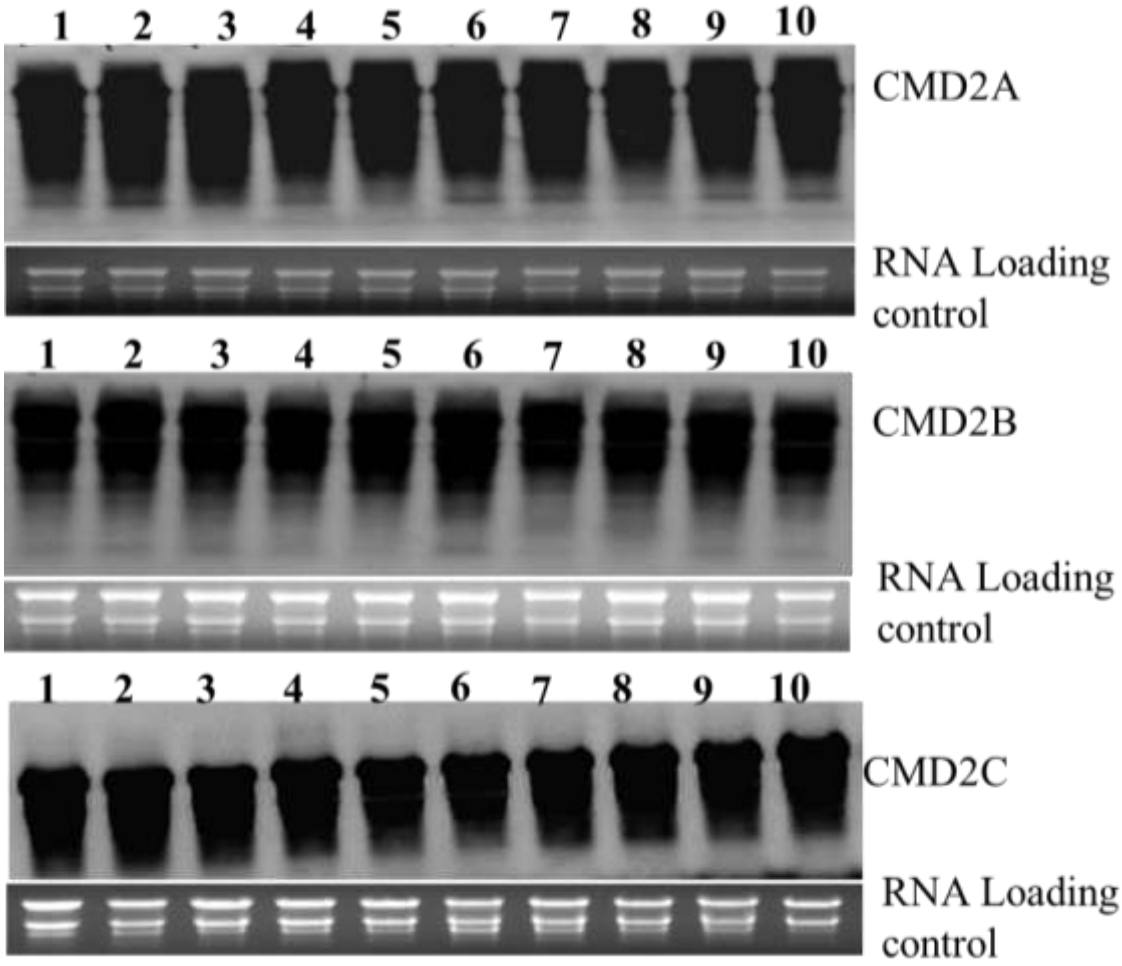


Figure 6. Northern blot analysis of putative CMD resistant gene messenger RNA accumulation in cassava. The following genotypes were analyzed; 1: TME3, 2: TME7, 3: TME204, 4: Oko-Iyawo, 5: TMS97/2205, 6: TMS98/0505, 7: TMS30001, 8: TMS30572, 9: 60444, 10: Ebwanatereka.

and NS169) are located 4.5 CentiMorgans (cM) and 7.1 cM from CMD2 locus (Parkes et al., 2015; Lokko et al., 2005; Akano et al., 2002). However, phenotypic data for these markers in F1 mapping population has not been reported (Okogbenin et al., 2013). Sixty eight percent of the total phenotypic variation of CMD2 is explained by SSRY28 at a distant of 13 cM from the CMD2 locus (Lokko et al., 2005; Akano et al., 2002), whereas NS198, that is reportedly linked with the CMD3 locus, explains 11% of variation in CMD resistance in F1 segregating populations (Okogbenin et al., 2012). One would expect that CMD resistant genotypes would contain 100% marker-trait association. However, CMD resistant loci have been described using eight molecular markers (Rabbi et al., 2014; Okogbenin et al., 2012; Lokko et al., 2005; Akano et al., 2002) that are sparsely distributed, making it impracticable to precisely associate flanking markers with CMD resistant status. High quality genome assembly and dense genetic maps are essential tools for mapping to allow identification of loci associated with traits (ICGMC, 2015; Gaur et al., 2015; Zhang et al., 2015). In soybean, for example, a high density genetic map saturated with 2500 molecular markers were developed to precisely map QTLs conferring resistance to *Fusarium graminearum* on 300 kb region of chromosome 8 (Acharya et al., 2015). Likewise, Zhang et al. (2015) developed linkage map containing 8007 markers to identify weeping trait in an ornamental woody plant *Prunus mume.* Such quality density maps are not be available for cassava.

SSR markers flanking the CMD2 and CMD3 loci, respectively were identified on chromosome 12 of cassava reference genome (AM560-2) (http://phytozome.jgi.doe.gov). SSR markers NS169 and NS158 sequences are both derived from the intron of the cassava gene designated Manes.12G074900.1 annotated to code for SWItch/Sucrose Non-Fermentable (SWI/SNF). This is part of an ATP-dependent gene family

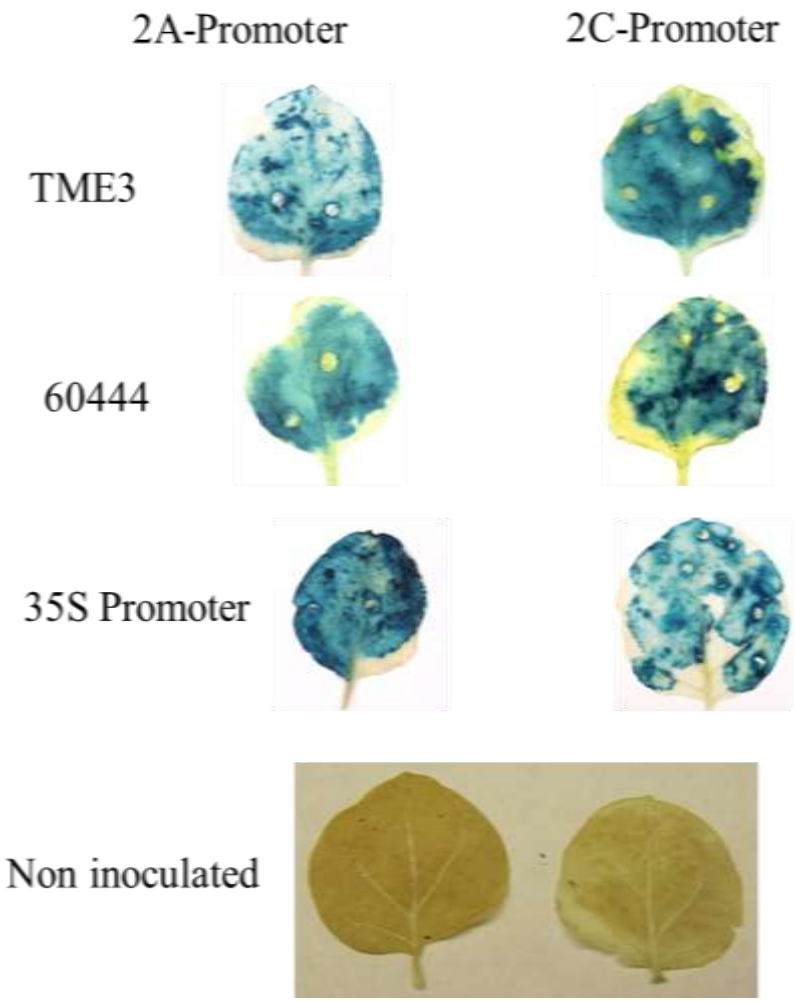


Figure 7. Transient expression of GUS reporter gene driven by endogenous promoter for native R genes in cassava when compared with 35S promoter.

found in both eukaryotes and prokaryotes that plays a central role in DNA packaging (Hargreaves and Crabtree, 2011). Conversely, marker SSRY28 is derived from cassava transcript Manes.12G074000.1 that codes for PREPHENATE DEHYDRATASE (P PROTEIN) involved in phenylalanine and tyrosine biosynthesis (Yoo et al., 2013; Maeda et al., 2011). CMD3 flanking marker NS198 is derived from cassava transcript Manes.12G016800.1 coding for protein phosphatase 2A (PP2A) involved in cell cycle control (Richard and Elder, 2005). The RME1 marker was shown to code for the partial motif of a disease resistance gene and mapped to several genomic regions. All three genes carrying sequences of SSR markers are key in the regulation of plant growth and development. As a result, the markers developed from such genes may not be sufficiently polymorphic to allow distinction between CMD susceptible and resistant genotypes. Single-strand conformation polymorphism (SSCP) of SSR markers on PAGE gel (Figure 1B) showed potential nucleotide differences in diverse cassava genotypes. Sequence analysis of SSRY28 revealed four nucleotide deletion at nucleotide position 114 in CMD resistant genotype TME3 but not in CMD susceptible genotype 60444.

In plants, it has been demonstrated that disease resistant genes are mostly located as clusters of homologous or heterologous sequences, and in some cases occur as both (Leister, 2004). Global analysis of the BAC clone sequences for the presence of disease resistance genes revealed NBS domains in each of the five BAC clones containing the RME1 fragment that mapped to Chromosome 12 (Table 3). For BAC29, a tandem repeat of NBS-LRR were mapped on chromosome 16 of cassava reference genome version 6. In the same chromosomal region, a cluster of 14 NBS-LRR genes were identified (Supplementary Figure 2). These NBS domains were homologous to NBS described in cassava genotypes TME3, TME117 and wild relative

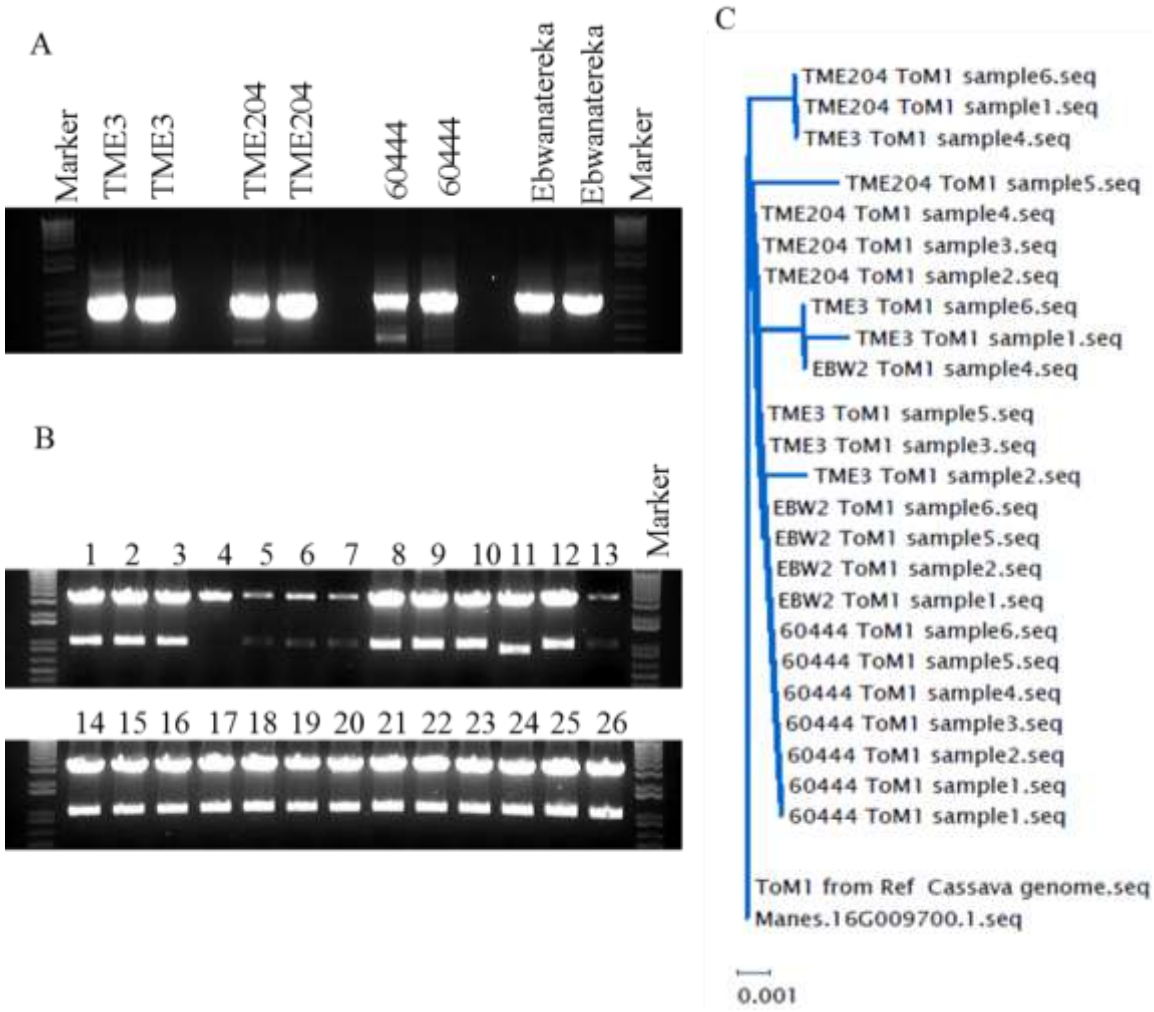


Figure 8. Tobamovirus multiplication protein 1 (TOM1) homolog in cassava. (A) RT-PCR amplification of TOM1 gene from cassava genotypes. (B) SacI and XbaI restriction digestion of TOM1 like genes from cassava CVs TME3, TME204, 60444 and EBW2 cloned into PCR Blunt II TOPO vector. Lanes: 1-6: TME3; 7-12: TME204; 13-20: 60444; 21-26: Ebwanatereka.

M. euprinosa and *M. brachyandra* (Gedil et al., 2012). The presence of numerous R genes in BAC29 corroborate with previous reports that indicate that R genes are usually present as clusters resulting from evolutionary pressure to counter changes in pathogen virulence proteins (Ashfield et al., 2012). In the same locus, the TOM1 homolog was identified, indicating a genomic region with high density of defense-related genes. Clusters of genes effective against bacteria, fungus, viruses and oomycetes have been mapped within the same vicinity on soybean chromosome 13 (Ashfield et al., 2012; Innes et al., 2008; Sandhu et al., 2005) and in chromosome 1 and chromosome 2 in lettuce (Christopoulou et al., 2015; McHale et al., 2009).

The number of disease resistant genes present in plant genomes varies greatly across species. For example, 143 R genes have been characterized in coconut (Puch-Hau et al., 2015) whereas cassava proteome has been reported to encode 28 Toll/interleukin-1 receptor Nucleotide-binding domain shared by apoptotic protease activating factor 1 (TIR-NB-ARC-LRR), 177 non-TIR-NB-ARC-LRR putative proteins, two with TIR-LRR domains (Soto et al., 2015). Other studies have identified 228 NBS-LRR genes present in the cassava reference genome, grouped into two subfamilies; 181 CC-domain-containing (CNL) and 47 TIR-domain-containing (TNL) (Lozano et al., 2015). A diverse cluster of 99 genes highly homologous to full-sized NBS-LRR genes have been identified in the reference genome encoding none, or only a small part, of the NBS domain (Lozano et al., 2015). The full length RME1 marker sequences are homologous to cassava genes encoding for NB-ARC (Figure 1). It can therefore be hypothesized that the RME1 marker is homologous to many endogenous disease resistant

genes in the cassava genome while the second RME1 allele (shorter 500 nt) would fit the truncated version of NB-ARC gene.

TME3 BAC library hybridization with RME1 fragment revealed 89 BAC clones positive for RME1 compared with 41 BAC clones for the three SSR markers, confirming a high number of R genes in cassava (Table 2). The RME1 full length sequence hits several places in cassava genome indicating that it is a highly repetitive sequence. This concurs with the fact that NBS-LRR are encoded by one of the largest gene families in plants carrying highly conserved NBS motifs (McHale et al., 2006). NBS-LRR motifs are in general conserved across many genes with pathogen resistance function (Hammond-Kosack and Parker, 2003; Glowacki et al., 2011).

Three full length and two truncated putative R genes occurring as tandem repeats were found in BAC29 sequences (Figure 4). This is in agreement with the recent report by Soto et al. (2015) who described the presence of clusters of full length and shorter versions of genes encoding NB-ARC-LRR in the cassava reference genome. Annotation of BAC29 and five other BAC clones mapping to the CMD2 locus genomic region and carrying RME1 marker sequences, revealed that putative R genes were localized in genomic region rich in transposable elements indicating a region undergoing fast evolution. This result fits with the model of an “arms-race” between pathogens and disease resistant genes that drives selective pressure to evolve R genes with specificity to new pathogen virulence proteins (Christopoulou et al., 2015). The truncated R genes identified in BAC29 showed a large deletion at three prime end (CMD2D) and a large insertion (7 kb) on CMD2e at nucleotide position 2237, indicating potential pseudogenes. Large deletions and insertions leading to frameshifts have been associated with condensed mRNAs and proteins. In most cases, disease resistant genes coding for partial NBS domain are regarded as pseudogenes (Luo et al., 2012). Truncated NBS-LRR with non-coding capacity have been located in genomic regions as clusters adjacent full length functional NBS-LRR in *Lotus japonicus, Medicago truncatula* and potato (Li et al., 2010; Lozano et al., 2012; Ameline-Torregrosa et al., 2008).

Cassava disease resistance genes have not been widely reported. Sequence analysis of R genes from different cassava genotypes revealed a high percentage of nucleotide identity indicating high levels of conservation (Figure 5B). Importantly, however, this was without association with presence or absence of functional CMD resistance loci. Resistance to CMD is governed by: single dominant gene(s) found within the CMD2 locus (Rabbi et al., 2014; Akano et al*.,* 2002), multigenic recessive resistance harbored by the CMD1 loci (Fregene and Puonti-Kaerlas, 2002) or synergism between CMD2 and an additional locus referred to as CMD3 (Okogbenin et al., 2012). Therefore, the identified NBS-LRR may not be involved in CMD resistance. Biolistic inoculation of cassava genotypes with cassava-infecting geminiviruses indicates that CMD resistance lacks the typical R gene mediated hypersensitive response (Patil and Fauquet, 2015). Studies by Louis and Rey (2015) identified resistance gene analogs (RGAs) encoding for resistance protein analogs (RPAs) that were uniquely overexpressed in TME3 recovering from South African cassava mosaic virus (SACMV) infection. However, none of the identified RGAs in TME3 localized with the CMD2 locus.

The cassava reference genome version 6.1 contains NBS-LRR clusters on chromosome 16 that are homologous to BAC29 and cassava isolated R genes. Northern blot analysis performed here indicated constitutive expression of BAC29 derived R gene in all diverse cassava genotypes (Figure 6). This may indicate their involvement in plant developmental functions while their role of antiviral defense remains to be demonstrated.

Through BAC end sequencing, 13 BAC clone libraries were constructed spanning regions around the CMD2 locus using the RME1 and BAC33b markers (CIAT, 2007). Full length BAC clone sequencing and assembly enabled construction of contigs of 100 kb in two independent clones while the shortest BAC contig assembled was 4.0 kb. However, these results indicated that some of the clones, for example BAC29, 52H02 and 52H13 were mapping to different genomic regions that have not been associated with CMD2 resistance. Approximately, 60% of reads derived from BACs such as 003H20, BAC33, BAC31 and BAC38 mapped to the *E. coli* genome, with the remaining 40% either not homologous to any cassava genomic region, or assembled poorly into a few short 1 – 2 kb contigs scattered throughout the genome. Other BACs contained sequence reads that did not map to cloning vector PIndigoBac536 BAC and had no hits within the cassava genome. Additional constrains encountered in using the TME3 BAC clone library were that detailed description of this library, such as position on physical map of cassava, BAC end sequences, and maps to physical clones in the freezer collection seems to be missing. These discrepancies observed in TME3 BACs studies here may have resulted from a mix-up of clones during library construction or processing. Another possibility that cannot be ruled out is low quality of the TME3 BAC libraries. Hybridization of markers to entire TME3 BAC libraries only provided clones originating from the marker region and did not form contiguous sequences that traversed through the CMD2 genomic region. Presence of *E. coli* sequences would also indicate contamination and poor quality of TME3 BAC clones library. This study therefore raises serious questions regarding the reliability of this TME3 BACs collection and the technical challenges of using it for identification of sequences such as those coding for CMD2 gene(s).

Conclusion

In this study, a BAC library was used in an attempt to study and identify genes residing within the CMD2 locus. CMD2 locus was mapped with four markers that did not show polymorphism in different cassava genotypes. Although, BAC libraries have been successfully utilized for map based cloning in other species, the present study was not able to identify clones spanning across the CMD2 locus, despite utilizing contig maps and markers previously reported. Sequences from some of the libraries reported in CMD2 region were found to be located in different chromosomal regions, certainly TME3 BAC library appears to be of insufficient quality at the CMD2 locus to be useful for identifying genes involved in this CMD resistance mechanism. It is likely that it would also not be a functional useful tool for discovery of other genes of importance in cassava. With regards to CMD, the situation is further compounded by the fact that the Cassava genome V6.1 contains gaps in CMD2 region making it difficult to design probes for screening potential BACs for chromosomal walking. Efforts should therefore focus on developing polymorphic molecular markers closely linked with the CMD2 locus that can easily be utilized to screen for CMD resistance. In addition, there is need for whole genome sequencing of CMD2 type cassava to identify sequences coding for genes harbored in CMD2 region. Transcriptome data should also be generated from CMD2 cassava genotypes to unravel the mode of action of CMD2 gene(s) and functional validation was performed through gene knock down or overexpression studies. New BAC libraries should be created from the same DNA source and be highly valuable is allowing accurate cloning of the gene(s) involved in CMD2 resistance.

Conflict of interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are grateful for financial support from Bill and Melinda Gates Foundation. They would like to thank Dr Luis Augusto and Dr Michael Atkins for providing BAC clones and Dr Steve Rounsley for sequencing.

REFERENCES

Acharya B, Lee S, Mian MR, Jun TH, McHale LK, Michel AP, Dorrance AE (2015). Identification and mapping of quantitative trait loci (QTL) conferring resistance to *Fusarium graminearum* from soybean PI 567301B. Theor. Appl. Genet. 128(5):827-838.

Akano A, Dixon A, Mba C, Barrera E, Fregene M (2002). Genetic mapping of a dominant gene conferring resistance to cassava mosaic disease. Theor. Appl. Genet. 105(4):521-525.

Akinbo O, Labuschagne M, Fregene M (2012). Increased storage protein from interspecific F1 hybrids between cassava (Manihot esculenta Crantz) and its wild progenitor (*M. esculenta* ssp. *flabellifolia*). Euphytica 185(2):303-311.

Ameline-Torregrosa C, Wang BB, O'Bleness MS, Deshpande S, Zhu H, Roe B, Young ND, Cannon SB (2008). Identification and characterization of nucleotide-binding site-leucine-rich repeat genes in the model plant *Medicago truncatula*. Plant Physiol. 146(1):5-21.

Asare PA, Galyuon IKA, Asare-Bediako E, Sarfo JK, Tetteh JP (2014). Phenotypic and molecular screening of cassava (Manihot esculentum Crantz) genotypes for resistance to cassava mosaic disease. J. Gen. Mol. Virol. 6(2), 6-18.

Ashfield T, Egan AN, Pfeil BE, Chen NW, Podicheti R, Ratnaparkhe MB, Ameline-Torregrosa C, Denny R, Cannon S, Doyle JJ, Geffroy V, Roe BA, Saghai Maroof MA, Young ND, Innes RW (2012). Evolution of a complex disease resistance gene cluster in diploid Phaseolus and tetraploid Glycine. Plant Physiol. 159(1):336-354.

Beyene G, Chauhan RD, Wagaba H, Moll T, Alicai T, Miano D, Carrington J, Taylor NJ (2015). Loss of CMD2-mediated resistance to cassava mosaic disease in plants regenerated through somatic embryogenesis. Mol. Plant Pathol.

Bi H, Aileni M, Zhang P (2010). Evaluation of cassava varieties for cassava mosaic disease resistance jointly by agro-inoculation screening and molecular markers. Afr. J. Plant Sci. 4(9):330-338.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI (2010). QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7(5):335-336.

Chauhan RD, Beyene G, Kalyaeva M, Fauquet CM, Taylor N (2015). Improvements in Agrobacterium-mediated transformation of cassava (*Manihot esculenta* Crantz) for large-scale production of transgenic plants. Plant Cell Tiss. Organ Cult. 121(3):591-603.

Christopoulou M, McHale L, Kozik A, Reyes-Chin Wo S, Wroblewski T, Michelmore RW (2015). Dissection of Two Complex Clusters of Resistance Genes in Lettuce (*Lactuca sativa*). Mol. Plant Microbe Interact. 28(7):751-765

CIAT (2006). Annual Report IP3. Improved cassava for the developing world, CIAT, Cali, Colombia.

CIAT (2007). Annual Report IP3. Improved cassava for the developing world, CIAT, Cali, Colombia.

Fregene M, Puonti-Kaerlas J (2002). Cassava biotechnology. Cassava: Biol. Prod. Util. 10:179-207.

Gaur R, Jeena Gaur G, Shah N, Gupta S, Pradhan S, Tyagi AK, Jain M, Chattopadhyay D, Bhatia S (2015). High density linkage mapping of genomic and transcriptomic SNPs for synteny analysis and anchoring the genome sequence of chickpea. Sci. Rep. P 5.

Gedil M, Kumar M, Igwe D (2012). Isolation and characterization of resistant gene analogs in cassava, wild Manihot species, and castor bean (*Ricinus communis*). Afr. J. Biotechnol. 11(85):15111-15123.

Glowacki S, Macioszek V, Kononowicz A (2011). R proteins as fundamentals of plant innate immunity. Cell. Mol. Biol. Lett. 16(1):1-24.

Hammond-Kosack KE, Parker JE (2003). Deciphering plant–pathogen communication: fresh perspectives for molecular resistance breeding. Curr. Opin. Biotechnol. 14(2):177-193.

Hargreaves DC, Crabtree GR (2011). ATP-dependent chromatin remodeling: genetics, genomics and mechanisms. Cell Res. 21(3):396-420.

Harimalala M, Chiroleu F, Giraud-Carrier C, Hoareau M, Zinga I, Randriamampianina JA, Velombola S, Ranomenjanahary S, Andrianjaka A, Reynaud B, Lefeuvre P, Lett JM (2015). Molecular epidemiology of cassava mosaic disease in Madagascar. Plant Pathol. 64(3):501-507.

Innes RW, Ameline-Torregrosa C, Ashfield T, Cannon E, Cannon SB, Chacko B, Chen NW, Couloux A, Dalwani A, Denny R, Deshpande S, Egan AN, Glover N, Hans CS, Howell S, Ilut D, Jackson S, Lai H, Mammadov J, Del Campo SM, Metcalf M, Nguyen A, O'Bleness M, Pfeil BE, Podicheti R, Ratnaparkhe MB, Samain S, Sanders I, Ségurens B, Sévignac M, Sherman-Broyles S, Thareau V, Tucker DM, Walling J, Wawrzynski A, Yi J, Doyle JJ, Geffroy V, Roe BA, Maroof MA, Young ND (2008). Differential accumulation of retroelements and diversification of NB-LRR disease resistance genes in duplicated regions following polyploidy in the ancestor of

soybean. Plant Physiol. 148(4):1740-1759.
International Barley Genome Sequencing Consortium (2012). A physical, genetic and functional sequence assembly of the barley genome. Nature 491(7426):711-716.
International Cassava Genetic Map Consortium (2015). High-Resolution Linkage Map and Chromosome-Scale Genome Assembly for Cassava (*Manihot esculenta* Crantz) from 10 Populations. G3 (Bethesda) 5(1):133-144.
Ishibashi K, Nishikiori M, Ishikawa M (2010). Interactions between tobamovirus replication proteins and cellular factors: Their impacts on virus multiplication. Mol. Plant Microbe Interact. 23(11):1413-1419.
Jørgensen K, Bak S, Busk PK, Sørensen C, Olsen CE, Puonti-Kaerlas J, Møller BL (2005). Cassava plants with a depleted cyanogenic glucoside content in leaves and tubers. Distribution of cyanogenic glucosides, their site of synthesis and transport, and blockage of the biosynthesis by RNA interference technology. Plant Physiol. 139(1): 363-374.
Kaweesi T, Kawuki R, Kyaligonza V, Baguma Y, Tusiime G, Ferguson ME (2014). Field evaluation of selected cassava genotypes for cassava brown streak disease based on symptom expression and virus load. Virol. J. 11(1):216.
Legg JP (1999). Emergence, spread and strategies for controlling the pandemic of cassava mosaic virus disease in east and central Africa. Crop Prot. 18(10):627-637.
Legg JP, Jeremiah SC, Obiero HM, Maruthi MN, Ndyetabula I, Okao-Okuja G, Bouwmeester H, Bigirimana S, Tata-Hangy W, Gashaka G, Mkamilo G, Alicai T, Kumar PL (2011). Comparing the regional epidemiology of the cassava mosaic and cassava brown streak virus pandemics in Africa. Virus Res. 159(2):161-170.
Legg JP, Kumar PL, Makeshkumar T, Tripathi L, Ferguson M, Kanju E, Ntawuruhunga P, Cuellar W (2015). Cassava virus diseases: Biology, epidemiology, and management. Adv. Virus Res. 91:85-142.
Leister D (2004). Tandem and segmental gene duplication and recombination in the evolution of plant disease resistance genes. Trends Genet. 20(3):116-122.
Li X, Cheng Y, Ma W, Zhao Y, Jiang H, Zhang M (2010). Identification and characterization of NBS-encoding disease resistance genes in *Lotus japonicus*. Plant Syst. Evol. 289(1-2):101-110.
Lokko Y, Danquah EY, Offei SK, Dixon AG, Gedil MA (2005). Molecular markers associated with a new source of resistance to the cassava mosaic disease. Afr. J. Biotechnol. 4(9):873-881.
Louis B, Rey C (2015). Resistance gene analogs involved in tolerant cassava–geminivirus interaction that shows a recovery phenotype. Virus Genes 51(3):393-407.
Lozano R, Hamblin MT, Prochnik S, Jannink JL (2015). Identification and distribution of the NBS-LRR gene family in the Cassava genome. BMC Genomics 16(1):360.
Lozano R, Ponce O, Ramirez M, Mostajo N, Orjeda G (2012). Genome-wide identification and mapping of NBS-encoding resistance genes in *Solanum tuberosum* group phureja. PLoS One 7(4):e34775.
Luo S, Zhang Y, Hu Q, Chen J, Li K, Lu C, Liu H, Wang W, Kuang H (2012). Dynamic nucleotide-binding site and leucine-rich repeat-encoding genes in the grass family. Plant Physiol. 159(1):197-210.
Maeda H, Yoo H, Dudareva N (2011). Prephenate aminotransferase directs plant phenylalanine biosynthesis via arogenate. Nat. Chem. Boil. 7(1):19-21.
Martin M (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet. J. 17(1):10.
McHale L, Tan X, Koehl P, Michelmore RW (2006). Plant NBS-LRR proteins: Adaptable guards. Genome Biol. 7(4):212.
McHale LK, Truco MJ, Kozik A, Wroblewski T, Ochoa OE, Lahre KA, Knapp SJ, Michelmore RW 2009. The genomic architecture of disease resistance in lettuce. Theor. Appl. Genet. 118(3):565-580.
Okogbenin E, Egesi CN, Olasanmi B, Ogundapo O, Kahya S, Hurtado P, Marin J, Akinbo O, Mba C, Gomez H, de Vicente C, Baiyeri S, Uguru M, Ewa F, Fregene M (2012). Molecular marker analysis and validation of resistance to cassava mosaic disease in elite cassava genotypes in Nigeria. Crop Sci. 52(6):2576-2586.
Okogbenin E, Moreno I, Tomkins J, Fauquet, CM, Mkamilo G, Fregene M (2013). Marker-Assisted Breeding for Cassava Mosaic Disease Resistance. Translat. Genom. Crop Breed.: Biotic Stress 1:291-325.
Okogbenin E, Porto MCM, Egesi C, Mba C, Espinosa E, Santos LG, Ospina C, Marín J, Barrera E, Gutiérrez J, Ekanayake I, Iglesia C, Fregene MA. (2007). Marker-assisted introgression of resistance to cassava mosaic disease into Latin American germplasm for the genetic improvement of cassava in Africa. Crop Sci. 47(5):1895-1904.
Parkes E, Fregene M, Dixon A, Okogbenin E, Boakye-Peprah B, Labuschagne M (2015). Developing Cassava Mosaic Disease resistant cassava varieties in Ghana using a marker assisted selection approach. Euphytica 203(3):549-556.
Patil BL, Fauquet CM (2009). Cassava mosaic geminiviruses: Actual knowledge and perspectives. Mol. Plant Pathol. 10(5):685-701.
Patil BL, Fauquet CM (2015). Studies on differential behavior of cassava mosaic geminivirus DNA components, symptom recovery patterns, and their siRNA profiles. Virus Genes 50(3):474-486.
Prochnik S, Marri PR, Desany B, Rabinowicz PD, Kodira C, Mohiuddin M, Rodriguez F, Fauquet C, Tohme J, Harkins T, Rokhsa DS, Rounsley S (2012). The cassava genome: current progress, future directions. Trop. Plant Biol. 5(1):88-94.
Puch-Hau C, Oropeza-Salín C, Peraza-Echeverría S, Gongora-Paredes M, Córdova-Lara I, Narvaez-Cab M, Zizumbo-Villarealb D, Sáenz-Carbonell L (2015). Molecular cloning and characterization of disease-resistance gene candidates of the nucleotide binding site (NBS) type from *Cocos nucifera* L. Physiol. Mol. Plant Pathol. 89:87-96.
Rabbi IY, Hamblin MT, Kumar PL, Gedil MA, Ikpan AS, Jannink JL, Kulakow PA (2014). High-resolution mapping of resistance to cassava mosaic geminiviruses in cassava using genotyping-by-sequencing and its implications for breeding. Virus Res. 186:87-96.
Ragupathy R, Rathinavelu R, Cloutier S (2011). Physical mapping and BAC-end sequence analysis provide initial insights into the flax (*Linum usitatissimum* L.) genome. BMC Genom. 12(1):217.
Richard YZ, T Elder R (2005). Viral infections and cell cycle G2/M regulation. Cell Res. 15(3):143-149.
Sandhu D, Schallock KG, Rivera-Velez N, Lundeen P, Cianzio S, Bhattacharyya MK (2005). Soybean Phytophthora resistance gene Rps8 maps closely to the Rps3 region. J. Hered. 96(5):536-541.
Shi X, Zeng H, Xue Y, Luo M (2011). A pair of new BAC and BIBAC vectors that facilitate BAC/BIBAC library construction and intact large genomic DNA insert exchange. Plant Methods 7(1):33.
Siritunga D, Arias-Garzon D, White W, Sayre RT (2004). Over-expression of hydroxynitrile lyase in transgenic cassava roots accelerates cyanogenesis and food detoxification. Plant Biotechnol. J. 2(1):37-43.
Soto JC, Ortiz JF, Perlaza-Jiménez L, Vásquez AX, Becerra LA, Mathew B, Léon J, Bernal AJ, Ballvora A, López CE (2015). A genetic map of cassava (*Manihot esculenta* Crantz) with integrated physical mapping of immunity-related genes. BMC Genom. 16(1):190.
Taylor N, Gaitan-Solis E, Moll T, Trauterman B, Jones T, Pranjal A, Trembley C, Abernathy V, Corbin D, Fauquet CM (2012). A high-throughput platform for the production and analysis of transgenic cassava (*Manihot esculenta*) plants. Trop. Plant Biol. 5(1):127-139.
Tomkins J, Fregene M, Main D, Kim H, Wing R, Tohme J (2004). Bacterial artificial chromosome (BAC) library resource for positional cloning of pest and disease resistance genes in cassava (*Manihot esculenta* Crantz). Plant Mol. Biol. 56(4):555-561.
Vanderschuren H, Moreno I, Anjanappa RB, Zainuddin IM, Gruissem W (2012). Exploiting the combination of natural and genetically engineered resistance to cassava mosaic and cassava brown streak viruses impacting cassava production in Africa. PloS One 7(9):e45277.
Vezzi F, Del Fabbro C, Tomescu AI, Policriti A (2012). rNA: a fast and accurate short reads numerical aligner. Bioinformatics 28(1):123-124.
Wang W, Feng B, Xiao J, Xia Z, Zhou X, Li P, Zhang W, Wang Y, Møller BL, Zhang P, Luo MC, Xiao G, Liu J, Yang J, Chen S, Rabinowicz PD, Chen X, Zhang HB, Ceballos H, Lou Q, Zou M, Carvalho LJ, Zeng C, Xia J, Sun S, Fu Y, Wang H, Lu C, Ruan M, Zhou S, Wu Z, Liu H, Kannangara RM, Jørgensen K, Neale RL, Bonde M, Heinz N, Zhu W, Wang S, Zhang Y, Pan K, Wen M, Ma PA, Li Z, Hu M, Liao W, Hu W, Zhang S, Pei J, Guo A, Guo J, Zhang J, Zhang Z, Ye J, Ou W, Ma Y, Liu X, Tallon LJ, Galens K, Ott S, Huang J, Xue J, An F, Yao Q, Lu X, Fregene M, López-Lavalle LA,

Wu J, You FM, Chen M, Hu S, Wu G, Zhong S, Ling P, Chen Y, Wang Q, Liu G, Liu B, Li K, Peng M (2014). Cassava genome from a wild ancestor to cultivated varieties. Nat. Commun. 5:5110.

Welsch R, Arango J, Bär C, Salazar B, Al-Babili S, Beltrán J, Chavarriaga P, Ceballos H, Tohme J, Beyer P (2010). Provitamin A accumulation in cassava (*Manihot esculenta*) roots driven by a single nucleotide polymorphism in a phytoene synthase gene. Plant Cell 22(10):3348-3356.

Wroblewski T, Tomczak A, Michelmore R (2005). Optimization of Agrobacterium-mediated transient assays of gene expression in lettuce, tomato and Arabidopsis. Plant Biotechnol. J. 3(2):259-273.

Yoo H, Widhalm JR, Qian Y, Maeda H, Cooper BR, Jannasch AS, Gonda I, Lewinsohn E, Rhodes D, Dudareva N (2013). An alternative pathway contributes to phenylalanine biosynthesis in plants via a cytosolic tyrosine: Phenylpyruvate aminotransferase. Nat. Commun. P 4.

Zhang HB, Scheuring CF, Zhang M, Zhang Y, Wu CC, Dong JJ, Li Y (2012). Construction of BIBAC and BAC libraries from a variety of organisms for advanced genomics research. Nat. Protoc. 7(3):479-499.

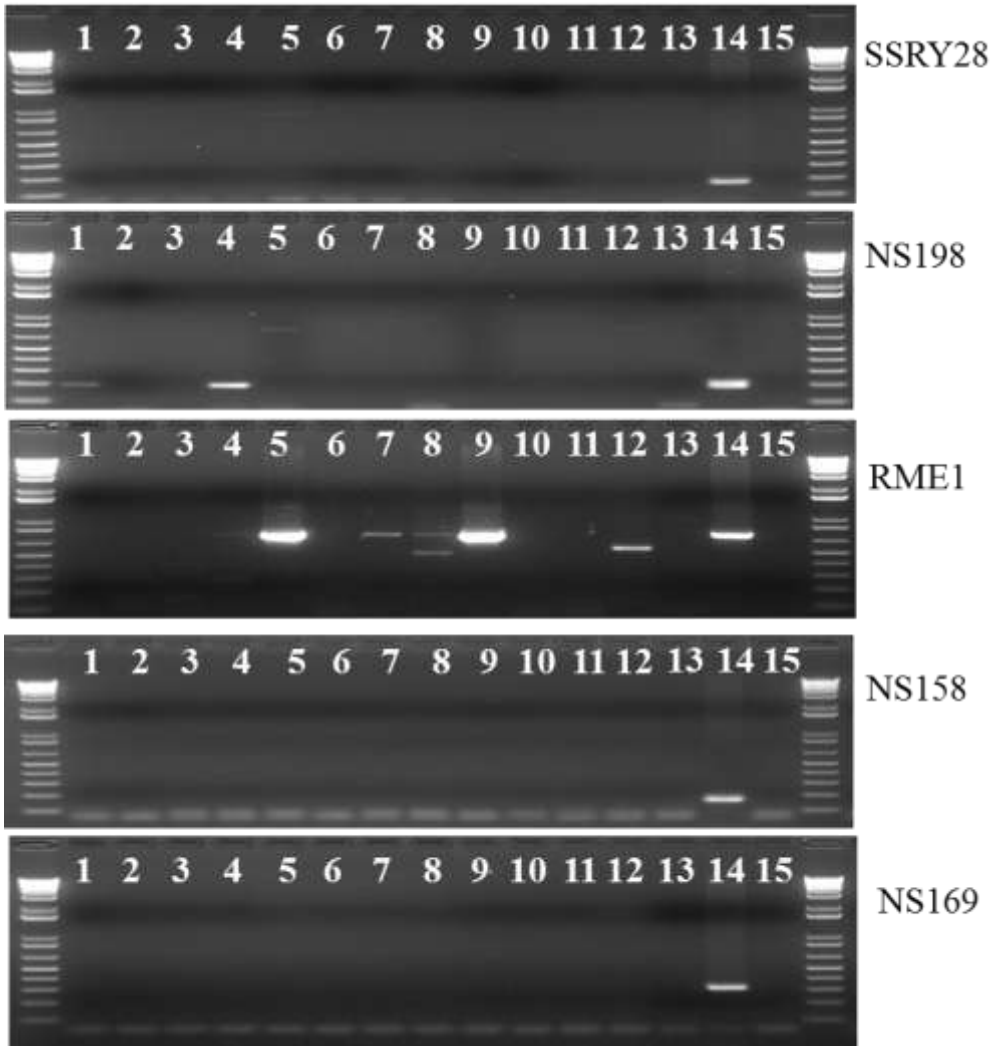
Zhang J, Zhang Q, Cheng T, Yang W, Pan H, Zhong J, Huang L, Liu E (2015). High-density genetic map construction and identification of a locus controlling weeping trait in an ornamental woody plant (*Prunus mume* Sieb. et Zucc). DNA Res. 22(3):183-191.

Supplementary Table 1. List of primers used in this study.

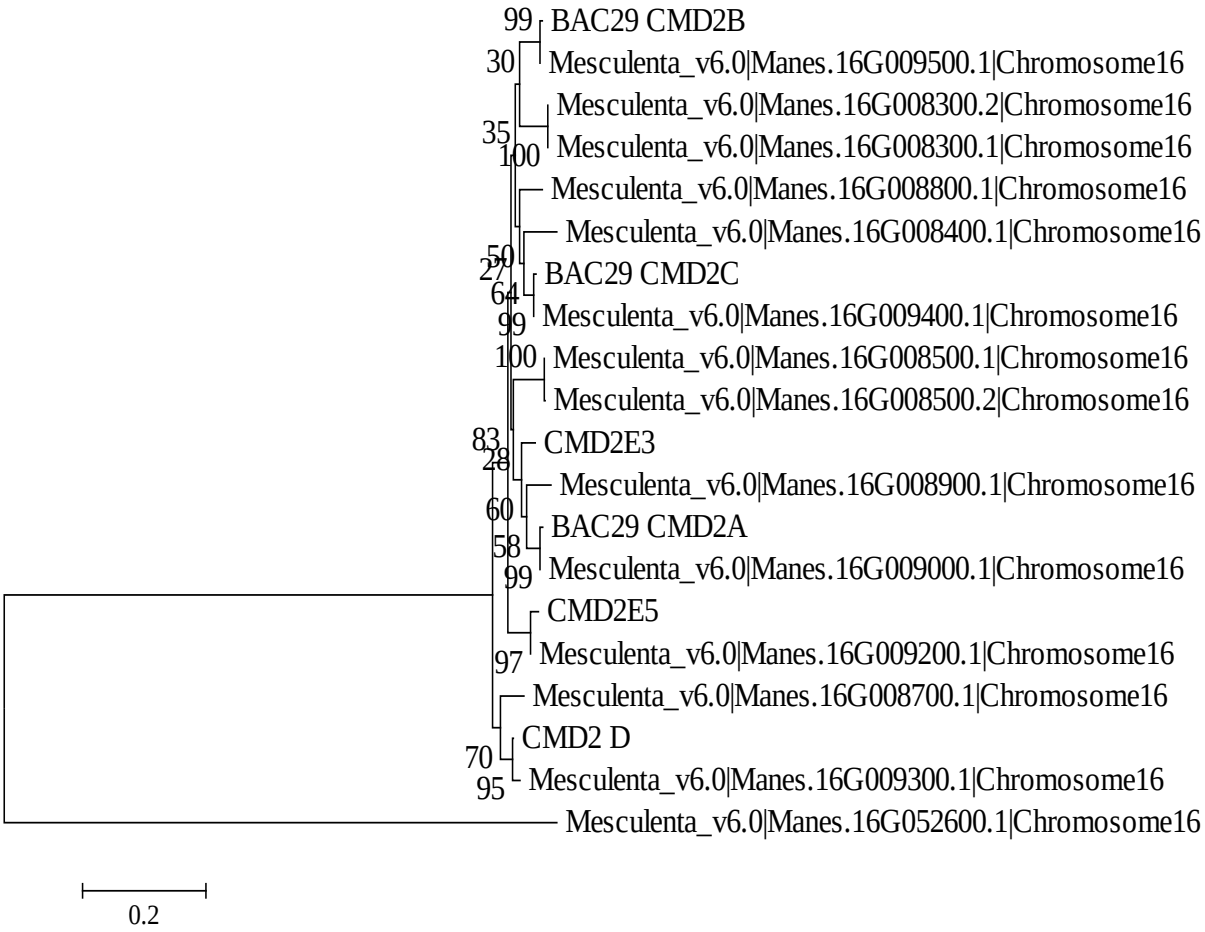
Primer name	Sequence (5' 3')
CMB2A R - KpnI	5' GGTACCTTTAATAGTACTAGATATGGCAGCACGC 3'
CMD2A F - SacI	5' GAGCTCTTCATTTAGTGCAAAATTCAAGTAATC 3'
CMD2B R2-SacI	5' GAGCTCCCATGATGCTATTTGCTGTCTCTTGCTGC 3'
CMD2B F2-XmaI	5' CCCGGGGAGCCAAGCATGATTTTACCTTTCGTGG 3'
CMD2C F - SacI	5' CGAGCTCAACTCAAAATTGAGTGATTCTAGCCG 3'
CMD2C R - KpnI	5' GGTACCATAAAGTATTCCATGCTGCCCATTTG 3'
CMd2A-prom-Sac II F	5' GACCGCGGAAGCTTCCATGTGCCTAAATTATTTAT 3'
CMd2A-prom- BamMHI R	5' GAGGATCCACCATGTCTGTGGACTTAACTAGTT 3'
CMD2B P SAC II - ASCI F- 1	5' GACCGCGGCGCGCCGAGAGGTACATTCATAATCCAATCCCCC 3'
CMD2B P BamMHI R - 2	5' GAGGATCCCCACGAAAGGTAAAATCATGCTTGGCTCTTCATC 3'
CMD2C P SAC II - ASCI F-1	5' GACCGCGGCGCGCCGTGCCTTGTATAGGAGGTTAGCATTG 3'
CMD2C P BamHI R - 2	5' GAGGGATCCCGGCTAGAATCACTCAATTTTGAGTTATG 3'
CMD2A ORF F XbaI	5' GATCTAGAATGGATGTTGTGACTTGTATCGC 3'
CMD2A ORF R BamHI	5' CTCGGATCCTTAAAATGATAAAACGGTACGTACTTCCTC 3'
CMD2B ORF F XbaI	5' GATCTAGAATGGATGTTGTGACTTGTATCGCTGG 3'
CMD2B ORF R BamHI	5' CTCGGATCCCAATTAAAATGCTAAAACGATATGTACCTCCTC 3'
CMD2C ORF F XbaI	5' GCTCTAGAATGGATGTTGTGACTTGTATCGCTGG 3'
CMD2C ORF R BamHI	5' CTCGGATCCCACAATTAAAATGCTAAAACGATACGTACCTC 3'
CMD2D F	5' CCACATCCAGTAACCTTAGATTAGTCAACTTCCC 3'
CMD2D R	5' GTTAAGTCCACGGACATGGATGTTGTGAGTTG 3'
CMD2.e.5 F	5' CCTGGGTGTGCCTACAGCACAAGTTGG 3'
CMD2.e.5 R	5' GTCCACAGACATGGATGTTGTGAC 3'
CMD2.e.3 F	5' TGAGAGAAGCAGCAAATGTC 3'
CMD2.e.3 R	5' GGGCTGGGCCTAACACTAGTGATTGTC 3'
CMD2.e.5 F1	5' GCTGATAGCCAGAGACAGTGATTCTCAGCAAG 3'
CMD2.e.5 R1	5' AATAGAGGGATTGACAACCTGAAAACATC 3'
CMD2abc 950R	5' AAGTCAGAGACTCAATCTCAGCGCCT 3'
CMD2abc 943F	5' AGAATGCAGGCGCTGAGATTGAGT 3'
CMD2a 1912R	5' TGGAAGAAGAGTCGCCATCAAGAG 3'
CMD2abc 1801F	5' GGGAGCTGAAGATGCTTGAAATTCT 3'
CMD2ab 2753R	5' TGTAGCTTGGGCAGATGCAACAAC 3'
CMD2a 2207F	5' AAGCCGGAGACGCCTTAGAGTAAA 3'
CMD2a 3177R	5' CGGTACGTACTTCCTCTTTGTTGTG 3'
CMD2c 1958R	5' CGGGACAGAGTAGACATAGCATTG 3'
CMD2c 2870R	5' CCCTTTCTGCAACAATTGCTTCTAAT 3'
CMD2bc 2500F	5' ATGCTTTGTTGCCGGAACTGGAAG 3'
CMD2C Promoter 253F	5' CGTTCATAATCCACTGCCCTTCCTCTTATTG 3'
CMD2C Promoter 1666R	5' CATGACATGGTTAACAGGCTGGAGTC 3'

Supplementary Table 2. Description of TME3 BAC clones hybridized with markers and tested with PCR.

Clone Number	Hybridized Marker	Markers associated with CMD2 locus			
		RME1	**SSRY28**	**NS158**	**NS169**
003H20	NS158/NS169/SSRY28	Positive	Negative	Positive	Negative
026L16	NS158/SSRY28	Positive	Negative	Positive	Negative
021C10	NS169/SSRY28	Positive	Negative	Negative	Negative
026N16	NS158/NS169/SSRY28	Positive	Negative	Positive	Negative
033J15	NS169/SSRY28	Positive	Negative	Positive	Negative
082G13	NS158/SSRY28	Negative	Negative	Negative	Negative
109O10	NS158/SSRY28	Negative	Negative	Negative	Negative
110N21	NS158/NS169/SSRY28	Negative	Negative	Positive	Negative
126O21	NS169/SSRY28	Positive	Negative	Negative	Negative
116N24	NS158/NS169/SSRY28	Positive	Negative	Positive	Negative
017N21	RME-1	Positive	Negative	Positive	Negative
022C04	RME-1	Negative	Negative	Negative	Negative
027O15	RME-1	Positive	Negative	Negative	Negative
092L05	RME-1	Positive	Negative	Positive	Negative
098J19	RME-1	Positive	Negative	Negative	Negative
102O06	RME-1	Positive	Negative	Positive	Negative
130D18	RME-1	Positive	Positive	Positive	Negative
136J08	RME-1	Positive	Positive	Positive	Negative
137O22	RME-1	Positive	Negative	Positive	Negative
146I10	RME-1	Positive	Negative	Positive	Negative
159C12	RME-1	Positive	Negative	Positive	Negative



Supplementary Figure 1. PCR analysis of thirteen BAC clones spanning across CMD locus region based on markers RME1 and BAC33b located on either side of the CMD2 locus using CMD2 mapping markers. The clones were represented as follows: 1, BAC12; 2, BAC16; 3, BAC21; 4, BAC22; 5, BAC23; 6, BAC26; 7, BAC29; 8, BAC31; 9, BAC33; 10, BAC38; 11, BAC40; 12, BAC44; 13, BAC45; 14, TME3 DNA; 15, negative control.



Supplementary Figure 2. Molecular phylogenetic analysis of NBS-LRR cluster in cassava chromosome 16 showing high nucleotide identities with BAC29 derived R genes.

African Journal of Biotechnology

Related Journals Published by Academic Journals

- *Biotechnology and Molecular Biology Reviews*
- *African Journal of Microbiology Research*
- *African Journal of Biochemistry Research*
- *African Journal of Environmental Science and Technology*
- *African Journal of Food Science*
- *African Journal of Plant Science*
- *Journal of Bioinformatics and Sequence Analysis*
- *International Journal of Biodiversity and Conservation*

academicJournals