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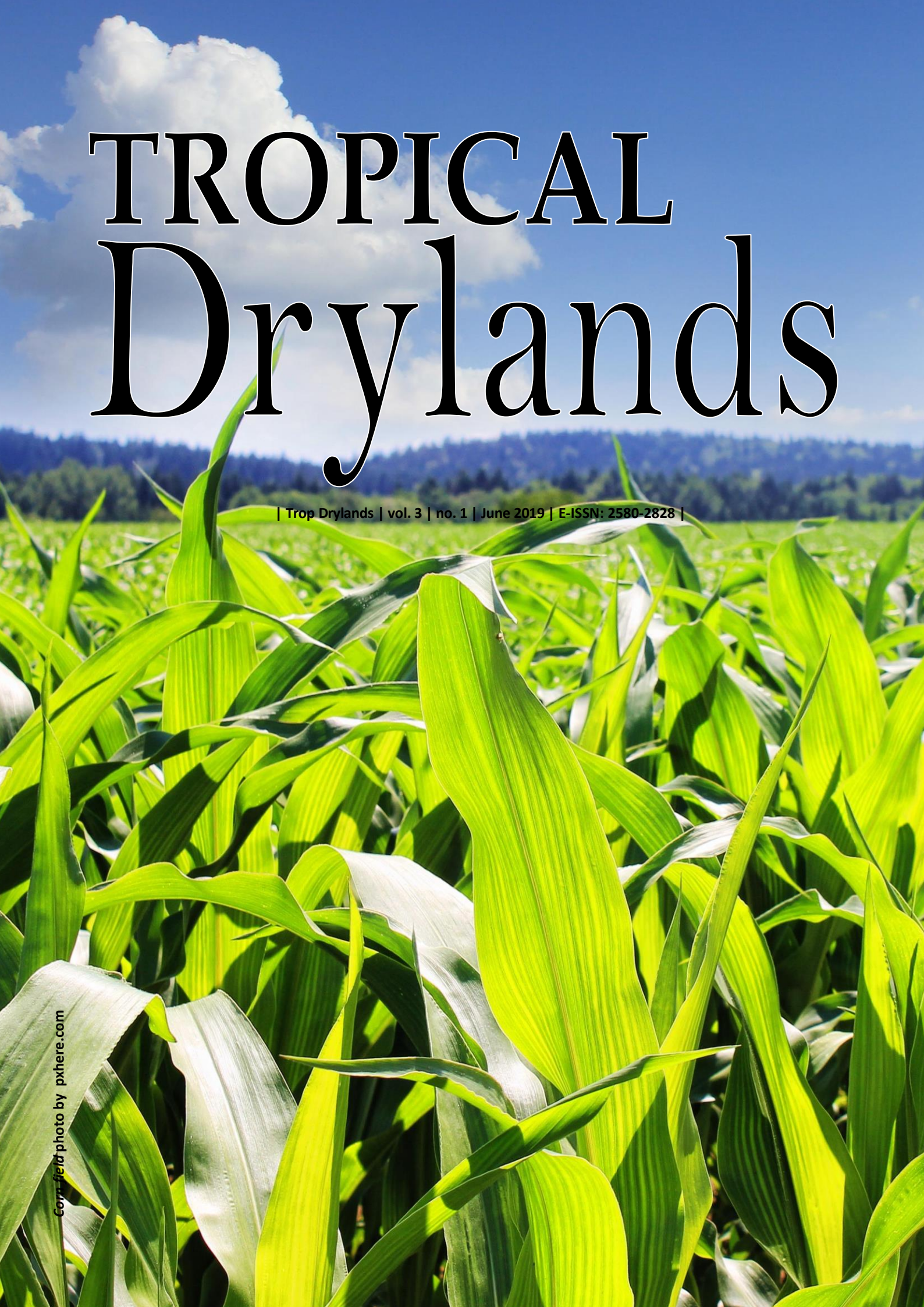

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Book:

Rai MK, Carpinella C. 2006. Naturally Occurring Bioactive Compounds. Elsevier, Amsterdam.

Chapter in book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds) Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta***.** 50th Annual Symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.) Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

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Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. www.molecularsystemsbiology.com. DOI:10.1038/msb.2008.24

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Maize bioengineering with c-repeat binding factor 1 (*CBF1*) as a technique for drought tolerance

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Abstract. *Kuria EK, Machuka J, Runo S. 2019. Maize bioengineering with c-repeat binding factor 1 (CBF1) as a technique for drought toleration. Trop Drylands 3: 1-10.* Africa is a drought-prone continent leading to failure in cultivation particularly to small-scale farmers who rely on rain-fed agriculture. Maize is the most widely cultivated main crop in Africa with more than 300 million people relying on it as their major staple food. Drought causes crop failure, famine and poverty and this is being aggravated by climate change. Therefore, it is necessary to develop drought tolerant maize. Since traditional propagation techniques are laborious and lengthy, bioengineering becomes a viable option. In this study, three maize inbred lines and one hybrid were altered with *CBF1* gene and appointed with mannose utilizing the Phosphomannose isomerase (*PMI)* gene. Genetic alteration was conducted through *Agrobacterium tumefaciens* and PCR was utilized to ascertain altered plants. Alteration frequency, alteration effectiveness and regeneration effectiveness were equated among the distinct genotypes altered. There were no remarkable dissimilarities in alteration frequency among the four maize genotypes. CML216 had the highest alteration effectiveness and regeneration effectiveness, followed by A188. No alleged transgenic plants were regenerated from TL27 and A188×TL18 under the circumstances implemented on account of their low regenerability. Further molecular analysis and drought stress tentatives on the expanded transgenic maize are significant prior to commercial release. Availability of drought tolerant maize would bring positive outcomes to fight famine, particularly in Africa.

Keywords: Bioengineering, corn, c-repeat binding factor 1, CBF1, desiccation toleration

INTRODUCTION

Maize is the third most important cereal in the world after wheat and rice. Based on endosperm and kernel proportion, McCann (2001) stated that there are five maize phenotypes, namely sweet, pop, floury, dent and flint. All of these phenotypes are originated from a single ancestor, i.e. teosente, that occurred in Mexico. Teosente is a wild grass native to Mexico and Central America (Doebly 2004). In general, its appearance is like maize but bears much longer lateral branches and produces only 5 to 12 kernels each secured tightly in a stony wrapper. Maize ear can bear about 500 or more naked kernels attached to the central axis of the ear called cob. The morphological dissimilarities between maize and teosente were hypothesized to have emerged from human screening during the domestication process (Doebly 2004). This hypothesis was verified to be true by Doebly (2004) who, through the utilization of quantitative trait loci (QTL) mapping, examined that five or six genes scattered throughout the genome were included in the evolution. These genes had quantitative impacts rather than distinct Mendelian impacts. It is the distinctive and epistatic impacts of these genes that altered teosente into maize.

Maize was brought into Africa by the Portuguese at the beginning of the 16th century (McCann 2001). It is important material for diet, fiber, oil, biofuel source and animal feed source, and is the main staple food for more than 300 million people in sub-Saharan Africa. Because of the combination of population growth, temperature increase due to global warming and successive decline of water table, there has been deficiency of maize supply while the demand for maize and its products is escalating. Between 2003 and 2005, the world food program spent US$ 1.5 billion to fight food deficiency due to drought and crop failure in sub-Saharan Africa alone (Edmeades 2008).

In both temperate and tropical regions, drought is the most significant abiotic stress, limiting maize cultivation. Therefore, it is necessary to develop drought-tolerant maize to fulfill the rising demand. This will increase land productivity capacity, price stability and may facilitate agricultural extensification which provides farmers with more cultivating options (Al-Abed et al. 2007). Drought is estimated to cause an annual loss in maize production of about 17% in the tropics and about 60% in regions such as southern Africa (Monneveux et al. 2006). Edmeades (2008) suggests that drought-tolerant maize will adapt to the variety in rainfall and temperatures which tend to be hotter and erratic. About 25% of crop losses caused by drought can be mitigated by genetic enhancement of drought tolerant genotypes (Edmeades 2008).

Traditional propagation practices have been greatly utilized to grow varieties accustomed to survive under abiotic and biotic stress. It is however laborious, lengthy and needs a lot of space. It is also hindered by the limited genetic variability within the genotypes (Ombori et al. 2008). Gene transition technologies are thus being utilized to complement conventional propagation practices. In

maize genetic modification, these technologies involve *Agrobacterium* mediated gene transition (Ishida et al. 1996), Polyethylene glycol (PEG) mediated alteration of protoplasts (Armstrong et al. 1990), microprojectile bombardment (Golovkin et al. 1993), and alteration via pollen tube pathway using a microsyringe (Zhang et al. 2005).

Though no drought tolerant transgenic maize has been commercialized so far, a lot has been conducted towards its establishment. Monsanto, an American-based multinational agricultural biotechnology corporation, is scheduled to start commercial sales of transgenic drought tolerant maize in the year 2012 (Edmeades 2008). The maize is expected to yield an average harvest enhancement of 15% under drought stress. Various studies on alteration of maize for drought tolerant types have been carried out. Shou et al. (2004) developed drought tolerant maize through expression of *Nicotiana* protein kinase 1 (*NPK1*), a tobacco mitogen activated protein kinase (MAPK) that activates genes securing photosynthesis process from breakage induced by drought and thus increasing harvest potential. Jeanneau et al. (2002) examined drought tolerant maize through overexpression of abscisic acid stress maturing protein (*ASRI*) and C4-phosphoenolpyruvate carboxylase (C4-PEPC), an enzyme that catalyzes the early fixation of atmospheric CO_2 even in high concentration of O_2 in relation to CO_2 due to the closing of stomata during drought. ASRI is an alleged transcription factor that has been suggested to govern transcription of genes that are regulated by sugar and abiotic stress in fruit and vegetative tissues (Shkolnik and Bar-Zvi 2008). Transgenic maize with raised expression of *Zea mays* nuclear factor Y B subunit (ZmNFYB2), a maize transcription factor analogous to *Arabidopsis thaliana* nuclear factor Y (NF-Y) that confers improved performance under drought circumstances, indicated tolerance to drought based on the reactions of stress-related parameters, such as chlorophyll content, stomatal conductance, leaf temperature, reduced wilting and maintenance of photosynthesis (Nelson et al. 2007).

Currently, a study is being carried out by a partnership called Water Effective Maize for Africa (WEMA) to develop drought tolerant maize through conventional plant propagation and genetic engineering. This project is predicted to increase maize productivity by more than 15% under moderate drought stress by the year 2017 (Edmeades 2008).

Arabidopsis CBF transcription factors interact with the drought responsive element (DRE), a *cis*-acting proponent element that governs gene expression in reaction to drought, brine and freezing stress (Al-Abed et al. 2007). In *Arabidopsis thaliana*, there are three *CBF* genes namely *CBF1*, *CBF2* and *CBF3* (Medina et al. 1999). These genes are also recognized as *DREB1b*, *DREB1c* and *DREB1a*, respectively. Members of this family of transcription factors hold a single APETALA2/ Ethylene responsive factor (AP2/ERF) DNA binding domain which strongly and specifically interacts with the DRE (Morran et al. 2007). Overexpression of these transcriptional factors, led by cauliflower mosaic virus 35S proponent, escalates stress toleration to freezing, drought and high brininess (Maruyama et al. 2009).

The objectives of this research was (i) To establish a gene construct comprising both *CBF1* and *PMI* genes; (ii) To alter Kenyan, tropical, temperate, and hybrid maize lines with *CBF1* gene through *Agrobacterium* mediated gene transition; (iii) To ascertain dissimilarities in alteration frequency, alteration effectiveness and regeneration effectiveness of the utilized distinct maize genotypes.

MATERIALS AND METHODS

Plant materials and explant source

One Kenyan line (TL27), one tropical line (CML 216), one temperate line (A188) and one hybrid (A188 ×TL18) were utilized for this study. The grains were cultivated in the field at the Plant Transformation Laboratory at Kenyatta University. To achieve fertilization phase, the plants were self-bred and unripe zygotic embryos excised 12-16 days after fertilization based on achievement of convenient embryo size. These unripe embryos were utilized as explants.

A188 was utilized due to its high alterability as informed by Ishida et al. (1996), Ishida et al. (2007), and Negrotto et al. (2000). It is a good administration for the alteration procedure and transgenic A188 can be crossed with other genotypes of preferable agronomic characteristics to treat drought tolerance to them. CML216 bears good combining capability for hybrid composition. It is immune to maize streak virus, *Erwinia turcicum* and *Puccinia sorgi*. It is late maturing and accustomed to tropical Africa (CIMMYT). This variety is highly regenerable as informed by Ombori et al. (2008). TL18 and TL27 are Kenyan lines accustomed to maize growing areas in the country. These genotypes were propagated at Kenya Agricultural Research Institute (KARI) and are tolerant to maize streak virus. Protocol for regeneration of these genotypes was explained by Ombori et al. (2008).

Establishment of gene construct

The plasmid pCAMBIA-CBF1 (Figure 1.A), acquired from Dr. Ron Mitler (University of Nevada), and pNOV 2819 (Figure 1.B) from Syngenta were utilized. The plasmids were altered into capable *E. coli DH5α* cells. Competent *E. coli DH5α* cells were arranged using calcium chloride technique as explained by Tu et al. (2005). For cloning, the two plasmids were refined from the *E. coli DH5α* cells using Qiagen plasmid miniprep kit (Hilden, Germany). Primers for amplification of *CBF1* gene cassette in pCAMBIA-CBF1 plasmid were developed with *SalI* and *Hin*dIII impediment sites integrated at the ends of the forward and reverse primers respectively (Table 1). *CBF1* gene cassette was then amplified using high fidelity taq DNA Polymerase (Fermentas, Austria). The PCR product was refined using Qiagen PCR refinement kit (Hilden, Germany) and absorbed sequentially with *SalI* and *Hin*dIII impediment enzymes (New England Biolabs, Ipswich, USA) to generate sticky ends. This insert was ligated to

pNOV 2819 vector which had been linearized using the same two enzymes used on the insert. pNOV 2819 carries the *PMI* gene that is significant for picking up altered plants on mannose comprising media. The resultant plasmid, named pNOV 2819-CBF1 (Figure 1.C), was altered into *Agrobacterium tumefaciens* strain *EHA101* which was used to infect the explant material. *CBF1* and *PMI* gene-specific primers (Table 1) were developed and utilized to ascertain the existence of the genes after alteration of *E. coli*, *Agrobacterium* and maize.

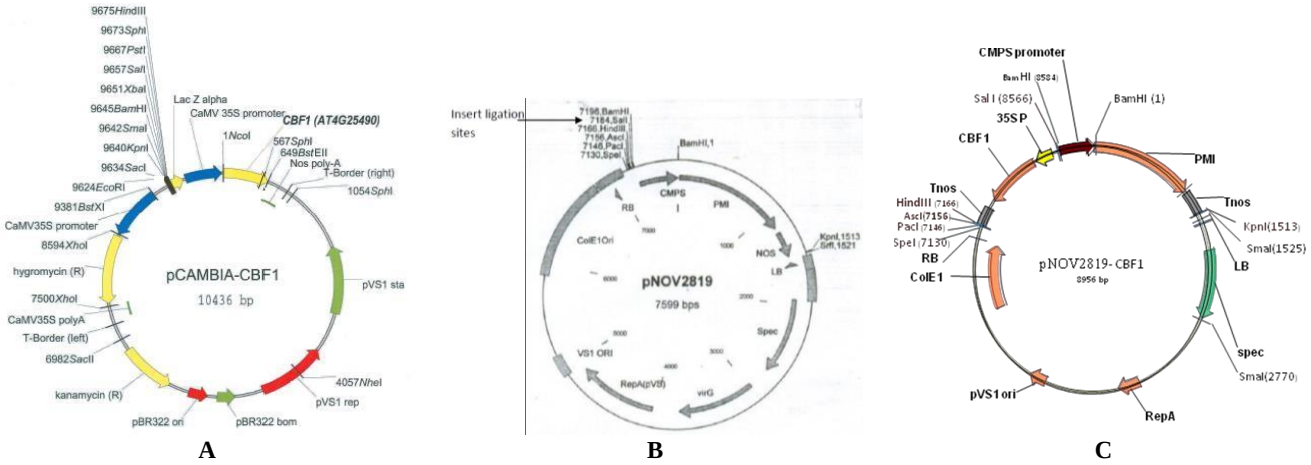
Alteration of *Agrobacterium tumefaciens*

The *Agrobacterium* strain *EHA101* was initiated from stock plates stockpiled for less than two weeks at 4ºC and preserved on LB medium (Duchefa, Netherlands) enhanced with 100 mgl^{-1} kanamycin. The *Agrobacterium* became capable to accept DNA through calcium chloride technique as defined by Tu et al. (2005), with the following modifications: (i) The cells were evolved at 28° C for all processes that needed brooding for accretion. (ii) The cells were evolved for 5 h after refreshing contrary to 2 h. (iii) After reaping, the cells were first resuspended in 0.15M $NaCl_2$ and hatched on ice for 15 min before 0.1M $CaCl_2$ was appended as in alteration of *E. coli* cells. (iv) After alteration process the cells were hatched for 1.5 h on LB medium before plating on selective plates comprising 100 mgl^{-1} kanamycin, and 100 mgl^{-1} spectinomycin. (v) Positive colonies were sifted after three days contrary to the 12 h needed for *E. coli* cells.

The process of maize alteration

Between 12-16 days after fertilization, distinct maize ears with unripe embryos of adequate size were reaped. The size of embryos was ascertained while the plant was still on the field. The maize ear was opened up a little to reveal the kernels. A spatula was utilized to detach an embryo and if the size was 1-1.2 mm in length along embryo axis, the ear was reaped and used quickly for alteration (Ishida et al. 2007).

In detaching the husk, the ears were surface sterilized by immersing in 2.5% sodium hypochlorite comprising two drops of tween 20 for 20 min. The cobs were then cleansed 3 times with hygienic refined water under aseptic circumstances in a tissue culture hood. A forceps speared the end of the ear on the silk scar side of the kernel to act as a handle while isolating the embryos. The top half of kernels was detached with a hygienic scalpel while the kernels were still embedded to the cob. The embryos were then pulled out with a spatula aseptically (Negrotto et al. 2000) and put in infection media.



A **B** **C**

Figure 1. A. pCAMBIA-CBF1 with *CBF1* gene and hygromycin opposition gene for picking up in plants; B. Vector (pNOV2819) with *PMI* gene and cloning sites for insertion of *CBF1* gene; C. Construct pNOV 2819-CBF1 carrying *PMI* and *CBF1* gene cassettes between the right and the left border

Table 1. Primers for amplifying *CBF1* gene cassette, *CBF1* and *PMI*

Primer	Sequence
35S forward (with *salI* site)	5’ TATCACA**GTCGAC**TGAGACTT 3’
Tnos reverse (with *Hin*dIII site)	5’ GCC**AAGCTT**TCCCGATCTAGTAACATAGAT 3’
CBF1 forward	5’ ATGAACTCATTTTCAGCTTTTTCTG 3’
CBF1 reverse	5’ TTAGTAACTCCAAAGCGACACG 3’
PMI forward	5’ ACAGCCACTCTCCATTCA 3’
PMI reverse	5’ GTTTGCCATCACTTCCAG 3’

Agrobacterium cultures were evolved for three days at 28° C on LB solid medium enhanced with 100 mgl^{-1} kanamycin, and 100 mgl^{-1} spectinomycin. One full loop of bacteria culture was scraped from the plate and dispersed in 5 ml of liquid infection medium in a 50 ml falcon tube. The tube was fixed horizontally on a bench-top shaker and shaken at 200 rpm for four h at 28° C. The unripe zygotic embryos from one ear were dissected and placed in bacteria-free LS infection media enhanced with 100 µM acetosyringone in hygienic petri dish. This media was then aspirated out and 20 ml of *Agrobacterium* suspension (OD_{550}= 0.3-0.4) was appended to the embryos. This plate was shielded with aluminum foil to allow darkness for effective *Agrobacterium* infection and left for infection for 10 min.

Infected embryos were moved to the surface of co-farming medium and excess *Agrobacterium* suspension was aspirated off the medium surface. Embryos were oriented with the embryo-axis side in contact with the medium and scutellum side up as recommended by Negrotto et al. (2000). These embryos with *Agrobacterium* were stored in the dark for three days at 18° C and then moved to LS resting medium enhanced with 1.8 mgl^{-1} silver nitrates and 250 mgl^{-1} carbenicillin and kept at 28° C in the dark for 10 days to establish callus (Negrotto et al. 2000).

The generated embryogenic tissues established unripe embryos and were moved to mannose comprising LS picking up media and cultured for 4 weeks with a subculture after 2 weeks. For A188, the first two weeks of picking up were conducted on media comprising 10 gl^{-1} mannose and 5 gl^{-1} in the subsequent two weeks as proposed by Negrotto et al. (2000). For the other genotypes, picking up was conducted at 5 gl^{-1} for the entire picking up period.

Surviving calli from picking up media were moved into regeneration media enhanced with 2.5 gl^{-1} mannose and placed under light at 28° C for 2 weeks. The generated green tissues were then moved to regeneration 2 medium without accretion regulators for two weeks and then to regeneration 3 medium comprising half strength of macro and micronutrients. The regenerated plants were acclimatized to soil and evolved in the greenhouse in accordance with the protocol by Ishida et al. (2007).

Analysis of transgenic plants

Putative transgenic plants were sifted for the existence of *CBFI* and *PMI* genes through PCR analysis. Leaf genomic DNA was isolated in accordance with CTAB extraction technique as defined by Zidani et al. (2005) with various modifications as defined below: About 5 g of leaf sample was cut from the alleged transgenic plant, immediately covered with aluminum foil and quickly chilled in liquid nitrogen in cryogenic tank where they remained until DNA isolation was conducted. Leaf tissues were detached from the tank and quickly crushed with a pestle in mortar comprising 4 ml of CTAB extraction buffer (100 mM Tris-HCL, 1.4 M NaCl, 20 mM EDTA) at 65°C. The liquid mixture was moved to a 10 ml falcon tube and hatched at 65° C in a water bath for 30 min. As much as 4ml of chloroform isoamyl alcohol (24:1) was appended to the samples and inverted twice to mix. The mixture was centrifuged at 5500 rpm for 10 min after which 1 ml of the aqueous layer was moved to 2 ml Eppendorf tube carefully. To precipitate DNA, 0.7 ml of isopropanol (stored at-20° C) was appended to each sample and inverted twice to mix. The tubes were chilled at -20°C for 20 min and then centrifuged at 5500 rpm for 15 min. The supernatant was discarded, 1 ml of 70% ethanol appended to rinse the DNA pellet and centrifuged at 12000 rpm. The supernatant was discarded and the DNA pellet air was desiccated for 30 min after which 100 µl of water was appended to liquefy the pellet and the liquefied DNA was stored at 4°C.

The result of the PCR was ascertained through agarose gel electrophoresis where 5 µL of the DNA was blended with 1 µL of ×6 gel loading dye (New England Biolabs, Ipswich, USA) and 1 µL of Sybr green gel loading dye (Invitrogen, USA) before loading. Electrophoresis was conducted at 100 volts for 40 min utilizing 0.8% agarose in ×1 TAE buffer and 1kb ladder (New England Biolabs) as the standard. Gel documentation equipment was utilized to visualize and record the gel.

Data analysis

Dissimilarities in alteration frequency, alteration effectiveness and regeneration effectiveness between genotypes were analyzed using ANOVA at 95% confidence interval. Alteration frequency was enumerated as the percentage number of mannose resistant callus events per number of embryos infected. Alteration effectiveness was enumerated as the percentage number of PCR positive events per total number of alleged transgenic events acquired and tested. Regeneration effectiveness was enumerated as the percentage number of shoots established per number of calli put on regeneration media.

RESULTS AND DISCUSSION

Preparation of plasmid construct

The awaited fragment containing the *CBF1* gene cassette was amplified from the plasmid pCAMBIA-CBF1. This insert was refined, absorbed with *SalI* and *Hin*dIII impediment enzymes to generate sticky ends and utilized for ligation. This insert was 1675 bp long as ascertained through agarose gel electrophoresis as indicated in Figure 2.A. The vector pNOV 2819 was linearised using *SalI* and *Hin*dIII impediment enzymes. Linearised plasmid generated a sharp band after electrophoresis while circular plasmid generated a thick band as indicated in Figure 2.B.

Proper ligation of the *CBF1* gene cassette insert to pNOV2819 vector was obtained through PCR and impediment digestion. These results were ascertained through agarose gel electrophoresis. On double digestion with *Hin*dIII and *KpnI* impediment enzymes, the awaited fragments of 3468 bp (consisting of the *PMI* and *CBF1* gene cassettes or the T-DNA) and 5671 bp (consisting of the remaining part of pNOV-2819 plasmid) were released as indicated on the gel picture in Figure 2.C.

Induction of callus from infected maize unripe embryos

Only slight increase in size was discovered on infected unripe zygotic embryos after being on co-farming media for 3 days. However, while on resting media, callus establishment happened. The embryos swell within the first two days before proliferation of cells to establish some calli by the fourth day. Both the compact type I and friable type II calli were established from the distinct maize genotypes. It was discovered that TL27, and A188×TL18 and had more cases of poor callus establishment. A188×TL18 established more watery non-embryogenic calli while TL27 established more rhizogenic calli (Figure 3).

Selection of transformants on mannose comprising media

Selection of altered calli from unaltered ones was clearly discovered after the first two weeks of picking up media. Transformed calli expanded vigorously on picking up media while unaltered calli remained small, turned brown and eventually dying by the fourth week of picking up. Putatively altered callus (x) grew vigorously while unaltered callus (y) remained small and brown (Figure 4).

Regeneration of callus into plants

Matured somatic embryos established on calli turned green within the first two days of exposure to light and shoots were established within a week (Figure 5). This was followed by root composition on regeneration 3 media though some plants established roots while still on regeneration 2 media.

Hardening and acclimatization of alleged transgenic plants

The alleged transgenic plants were well hardened and acclimatized on autoclaved peat moss. They were first carefully removed from the media taking care not to break the roots. The roots were washed with hygienic water to detach any media attached to them, planted on peat moss and shielded with polythene bag for 3 days to prevent death from excessive evapotranspiration as indicated in Figure 6.A. These plants were then moved to soil after two weeks and evolved to maturity in the greenhouse (Figures 6.B and 7).

Molecular analysis of alleged transgenic maize

As ascertained through agarose gel electrophoresis on isolated maize genomic DNA, high concentration of good quality DNA for further molecular analysis was acquired (Figure 8). PCR analysis utilizing *PMI* gene specific primers uncovered the existence of the *PMI* gene on most of the transgenic plants (Figure 9). DNA from the alleged transgenic plants was also subjected to PCR utilizing *CBF1* gene specific primers to ascertain the existence of *CBF1* gene and most were positive.

Gel picture in Figure 10 shows *CBF1* PCR for some seven T_0 transgenic plants and an unaltered plant as a negative control. pNOV-CBF1 plasmid was utilized as a positive control.

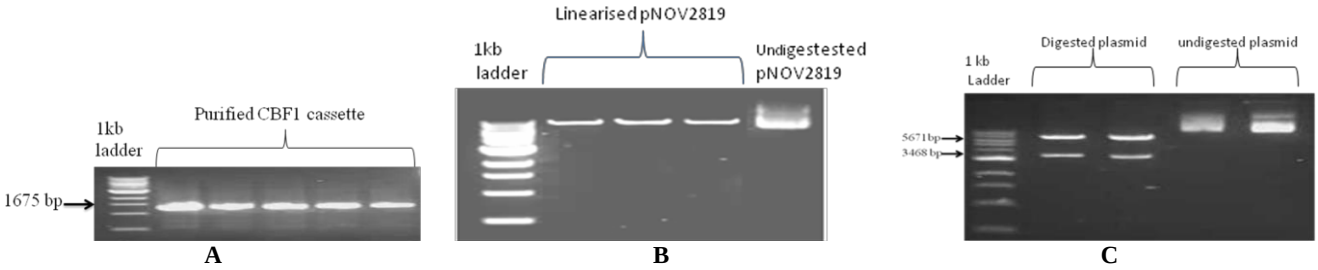


Figure 2. A. *CBF1* gene cassette amplified from pCAMBIA-CBF1 plasmid; B. linearised pNOV2819 after digestion with *SalI* and *Hin*dIII in comparison to undigested vector; C. Validation of pNOV-CBF1 plasmid through impediment digestion with *Hin*dIII and *KpnI*

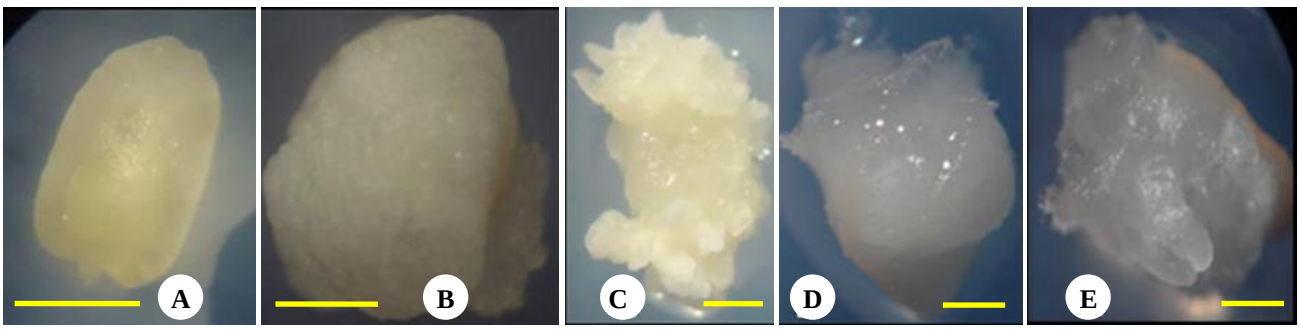


Figure 3. Callus composition from CML 216 maize embryos. Immature zygotic embryo excised from a cob (A). Type I callus (B). Type II friable callus (C). Watery non-embryogenic callus (D). Rhizogenic callus forming root-like structures (E) (bar = 1 mm)

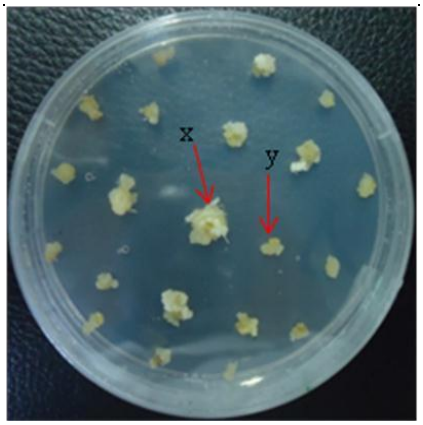


Figure 4. A188 calli on picking up media presenting putatively altered callus (x). Untransformed callus (y) after three weeks on picking up media. (bar = 1 cm)

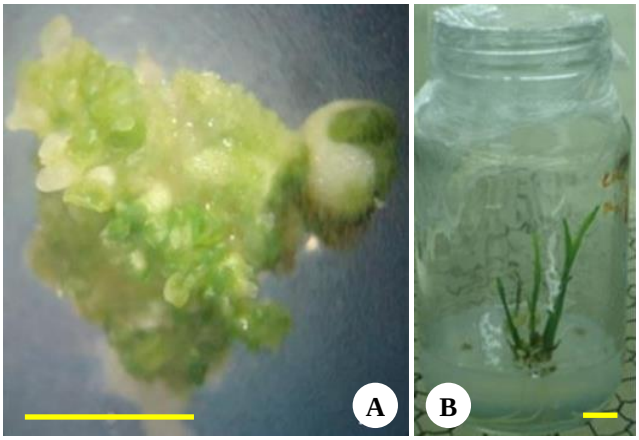


Figure 5. CML 216 Callus on regeneration media turning green and forming shoots on exposure to light (A). Established shoots and roots on regeneration media (B) (bar = 1 cm)

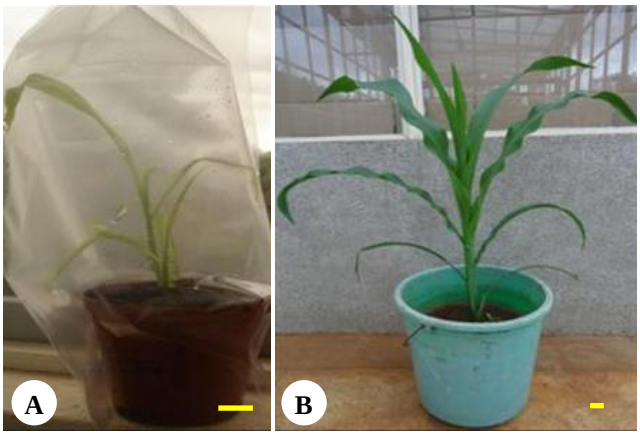


Figure 6. Hardening and acclimatization of alleged transgenic CML216 plants: Hardening a plant on peat moss (A). Hardened plant on soil in the glasshouse (B) (bar = 2 cm).

Figure 7. Mature CML 216 transgenic plant (line 3) with normal tassel and ears. (bar = 10 cm)

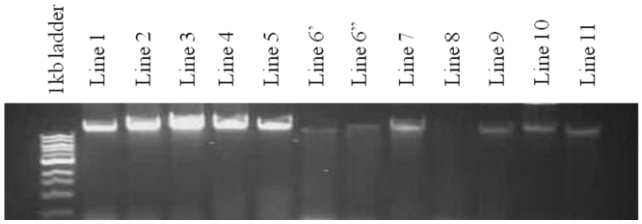


Figure 8. Gel electrophoresis of refined maize genomic DNA, presenting high concentration of good quality DNA acquired from putatively transgenic plants

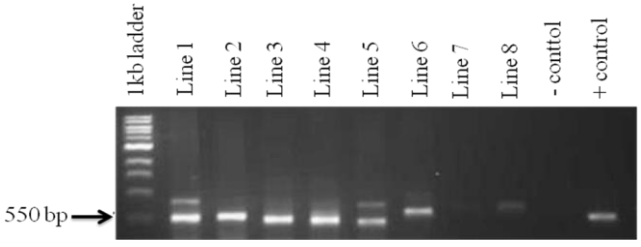


Figure 9. PCR utilizing PMI gene specific primer on maize genomic DNA; lines 1 and 2; putatively transgenic A188, lines 3-8; putatively transgenic CML 216; Negative control was unaltered maize DNA and Positive control was PNOV 2819 plasmid DNA

Exhibition of somaclonal variety by alleged transgenic plants

Though most T_0 plants indicated normal establishment, a few abnormal characteristics were discovered most probably as a result of somaclonal variety. These characteristics involved dwarf plants, outturn of silk and setting of grains at the tassel, folding/drooping downwards during flowering phase and outturn of multiple cobs on one node (Figure 11).

Alteration and regeneration

Though mannose tolerablish calli were discovered in all the genotypes, representing triumphant alteration, only A188 and CML216 regenerated to give putatively transgenic plants (Table 2). There was no remarkable dissimilarity discovered in alteration frequency among the four maize genotypes utilized in this study after one way ANOVA at 95% confidence interval of the mean.

An analysis of variance (ANOVA) uncovered remarkable dissimilarity in the regeneration effectiveness (P=0.007) at 95% confidence interval of the mean (Table 3). Tukey's HSD post-hoc test indicated remarkable dissimilarity in the regeneration effectiveness of CML216 equated to TL27 (P=0.010) and A188×TL18 (P=0.010).

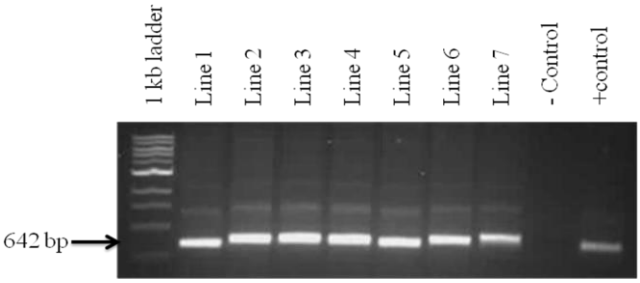


Figure 10. PCR utilizing CBF1 gene specific primers on maize genomic DNA; lines 1 and 2; alleged transgenic A188. Lines 3-7; alleged transgenic CML 216.-control was unaltered maize. +control was PNOV 2819-CBF1 plasmid

One way ANOVA indicated a remarkable dissimilarity in alteration effectiveness in the four maize genotypes at 95% confidence interval of the mean. Tukey's HSD post-hoc test indicated that CML216 and A188 had remarkable dissimilarity in alteration effectiveness at P=0.05 with TL27 and A188×TL18.

Table 2. Alteration and regeneration data

Genotype	Exp.	Number of embryos	TF (%)*	RE (%)*	TE (%)*
A188	1	280	36	0	0
	2	151	35	18	100
	3	187	69	61	90
CML216	1	175	19	91	87
	2	148	20	50	88
	3	179	39	129	87
TL27	1	50	12	0	0
	2	205	66	0	0
	3	125	8	0	0
A188×TL18	1	62	37	0	0
	2	200	31	0	0
	3	75	13	0	0

Note: TF: Alteration frequency RE: Regeneration Efficiency TE: Alteration effectiveness

Table 3. Analysis of variance for alteration frequency, regeneration effectiveness and alteration effectiveness

Para-meter	Genotypes A188	CML216	A188×TL18	TL27	P-value
TF*	46.67±11.17	26.00±6.51	27.00±7.21	28.7±18.7	0.592
RE*	26.3±18.1	90.0±22.8	0±0	0±0	0.007
TE*	63±31.8	87.33±0.33	0±0	0±0	0.009

Note: TF: Alteration frequency RE: Regeneration Efficiency TE: Alteration effectiveness

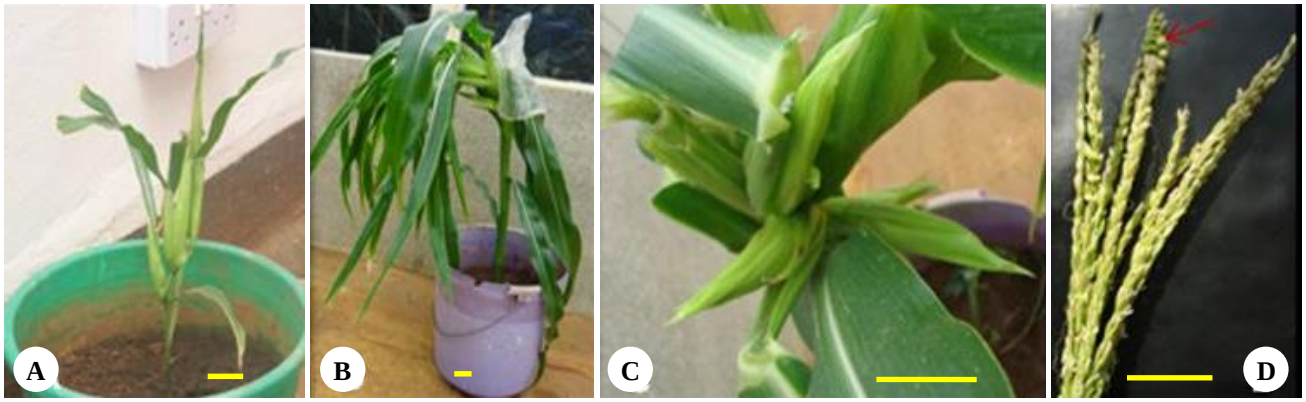


Figure 11. Somaclonal variety discovered on T_0 plants: A. Dwarf phenotype discovered in line 1. B. Drooping of shoot during the flowering phase (line 10). C. Multiple cobs generated on a single node (line 10). D. Setting of tassel grains (line 16). (bar = 5 cm)

Discussion

Maize alteration and regeneration

Transgenic maize was acquired through *Agrobacterium* mediated gene transition. Only two genotypes of maize, i.e. A188 and CML216, gave transgenic plants. No plants were regenerated from TL27 and A188×TL18. In spite of observation of altered callus from these genotypes that did well on mannose comprising picking up media, these genotypes were unable to regenerate under the utilized circumstances. This is ascribed to their poor regenerability.

Regeneration of TL27 and TL18 was informed by Ombori et al. (2008) at lower mean number of shoots per culture equated to CML216. It was predicted that a hybrid between the highly regenerable A188 as defined by Negrotto et al. (2000) and the lowly regenerable TL18 as defined by Ombori et al. (2008) would bear a higher regeneration frequency. This is based on findings of Bronsema et al. (1997) who informed that embryogenic capacity of maize unripe zygotic embryos is maternally heired. This was however not the case and probably this does not take place with the composite of the two genotypes. Failure to regenerate this hybrid and TL27 could also be on account of a genotype dependent interaction with *Agrobacterium* inherent in TL18 and TL27 alike to the hypersensitivity to *Agrobacterium* defined by Kuta and Tripathy (2005).

Determination of the right time to harvest the unripe zygotic embryos was complicated by their accretion being highly dependent on environmental circumstances and the seasonal nature of availability of quality unripe embryos. Once embryos achieved the right size, they could only be stored for a maximum of two days. In accordance with Ishida (2007), triumphant maize alteration is primarily dependent on the reaction of unripe embryos in tissue culture, the type of cells that grow from them and subsequent characteristics in accretion and regeneration. Quality of unripe embryos, unripe embryos establishment phase and circumstances utilized during alteration are also critical factors for triumphant alteration. To achieve these critical factors and to overcome the above mentioned challenges, only vigorously growing plants were utilized. Embryo size was ascertained while the cob was still attached to the plant and cultivating was conducted at regular intervals for continuous supply of embryos.

Embryogenic type I, type II and non embryogenic calli were established from the distinct maize genotypes utilized in this study. The composition of these embryo types has been informed by McCain (1988), Songstad et al. (1992), and Bronsema et al. (1997). Type I callus is compact on account of the somatic embryos being morphologically complex and organized while type II callus is friable on account of the somatic embryos being discretely organized throughout the cultures (Songstad et al. 1992). In accordance with McCain (1988), type I callus grows from fusion of aborted somatic embryos that arise directly from the scatellum base of the zygotic embryos or from continued accretion of the scatellum itself forming a callus. Inheritance of somatic embryogenesis and regeneration in maize includes two genes expressed in the abaxial region of the middle and basal side of the scutellum of unripe zygotic embryos. If these genes fail to proliferate only non embryogenic callus is established (Bronsema et al. 1997). Thus the composition of distinct types of calli in distinct maize genotypes is on account of their genetic properties.

The established non embryogenic calli involved the soft watery callus and the rhizogenic calli. The former changed into brown during culture and finally perished without establishing somatic embryos. Rhizogenic callus was generally discovered in TL27. This callus established many root-like structures and established no somatic embryos. Rhizogenic callus takes place when the callus meristem is established endogenously and its establishment is ascribed to genetic properties of distinct genotypes (Bronsema et al. (1997).

The Polymerase Chain Reaction (PCR) was effectively utilized to ascertain the alteration of *E. coli* cells, *Agrobacterium* cells and maize. Utilizing *PMI* and *CBF1* gene specific primers, the predicted fragment of 550 and 642 base pairs respectively were amplified from transgenic maize (Figure 9 and 10). Bands that were a little heavier than predicted were discovered in some events. Shou et al. (2004) ascribed this to some inefficiency in *Agrobacterium* mediated gene transition that brings about the unification of part of non T-DNA vector backbone along with the T-DNA. The level of this co-transition relies on the plant species altered, *Agrobacterium* strain and utilized alteration technique.

The terms alteration frequency and alteration effectiveness are utilized to delineate the triumph of a particular alteration tentative as a result of unification of the DNA into plants genome, propagation of altered cells and regeneration and description of alterants. Triumph of alteration is then ascertained by alteration capability of host cells, selectable marker utilized as some impede accretion of altered cells and the capability of a plant to regenerate from altered cells (Zuo et al. 2002). Alteration frequency mean of between 46% and 27% was discovered for the distinct genotypes (Table 3). For A188 the mean acquired (46%) was higher than that informed in Negrotto et al. (2000) of 30%. This dissimilarity could be ascribed to the time of infection of 10 min contrary to the 5 min utilized by Negrotto et al. (2000). Such high alteration frequency (45%) was achieved utilizing biolistics by Wright et al. (2001) when selecting maize on mannose.

A188 held a greater alteration frequency than the other genotypes, representing that it was more alterable. However, CML 216 indicated greater regeneration effectiveness than A188 in spite of its lower alteration frequency. The high alteration frequency of A188×TL18 and TL27 as equated to CML216 was not of considerable value as these genotypes had very low regeneration effectiveness and failed to give alleged transgenic plants. This agrees with Ombori et al. (2008) that the *in vitro* regeneration system is often the restricting factor in the practice of genetic alteration methods for crop enhancement of most maize genotypes.

The various abnormal phenotypes discovered in transgenic plants acquired such as dwarfism, drooping of shoot, composition of multiple ears in one node and tassel

grains (Figure 9) are primarily ascribed to somaclonal variety. This refers to genetic and phenotypic variety among clonally propagated plants of a single donor that occurs as a result of epigenetic changes (Kaeppler et al. 2000). Dwarf and plants of normal height that were positive for *CBF1* gene were acquired.

The dwarf phenotype could have been on account of somaclonal varieties as a result of tissue culture or overexpression of *CBF1* as informed by Hsieh et al. (2002) who recommended that heterologous expression of the gene affects establishment process in transgenic tomato plants. Similar impacts were informed in *Arabidopsis* by Kasuga et al. (1999) on overexpression of *CBF3* and associated this with the utilization of 35S cauliflower mosaic virus promoter which was also utilized in this study. Composition of tassel grains and dwarfism as a result of somaclonal variety was also discovered by Omer et al. (2008) during regeneration of Sudanese inbred lines and open bred varieties.

Triumph in further molecular analysis, drought stress and toxicology studies on these acquired transgenic maize genotypes would set the phase for the release of drought tolerant maize for commercialization. Drought tolerant plants would bring about raised productivity and price stability. This would bear a very considerable impact in decreasing famine and poverty particularly in sub Saharan Africa where maize is the main crop.

Conclusion

A plant alteration vector comprising drought tolerance increasing CBF 1 gene and PMI gene for picking up on mannose was established and utilized in alteration of four maize genotypes. Out of these, two genotypes were triumphantly altered and the acquired grains were utilized for further analysis of transgenic plants. There were no remarkable dissimilarities in alteration frequency among the genotypes utilized in this study. CML 216 was more regenerable than other genotypes as it indicated higher regeneration effectiveness. It was therefore found to be more appropriate for alteration under the circumstances utilized in this study. This is because the ability to regenerate is critical in plant alteration. Triumph in alteration of TL27 and A188×TL18 was restricted by their inability to regenerate under the circumstances that were utilized in this study. This is because these genotypes gave alteration frequency mean that was not significantly distinct from the others but their calli could not regenerate to give whole plants.

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The effect of vermiwash and vermicompost produced from leaves of *Azadirachta indica* and *Citrus aurantifolia* on the growth parameters of celery through hydroponic system

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[1]Department of Biology, Faculty of Natural Sciences, University of Guyana. Turkeyen Campus, Greater Georgetown, Guyana. Tel.: +592-222-5423, Fax.: +592-222-5122, ♥email: abdullah.ansari@uog.edu.gy
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Manuscript received: 3 December 2018. Revision accepted: 16 February 2019.

Abstract. *Ansari AA, Raghubeer H, Jaikishun S. 2019. The effect of vermiwash and vermicompost produced from leaves of* Azadirachta indica *and* Citrus aurantifolia *on the growth parameters of celery through hydroponic system. Trop Drylands 3: 11-16.* Hydroponic is a method of growing plants in water solution with additional elements including vermicompost and vermiwash generated from organic matters. This research was designed to determine the physicochemical composition of vermiwash and vermicompost produced from neem (*Azadirachta indica*) and lime (*Citrus aurantifolia*) and their influence on the growth parameters on celery (*Apium graveolens*) in hydroponic system. Plants were treated with six treatments-A (nutrient solution for hydroponic systems), B (vermiwash and vermicompost made from T_1), C-control (water), D (vermiwash and vermicompost made from T_2), E (vermiwash and vermicompost made from T_3). All treatments were replicated six times in a complete randomized block design (CRBD). Results indicated that vermiwash and vermicompost when used in combination can be substituted for chemical hydroponic solutions used in hydroponic systems. T2 had a significant effect on celery plants where it produced plants with greater heights and stem diameter. Maximum amount of sodium was seen in plants treated with F. Plants treated with T1 had the highest number of leaves and branches. This research will further enhance the need for hydroponic agriculture as it has less detrimental effects on the environment and produces greater yield and better quality of crops.

Keywords: Hydroponic, plant nutrients, physicochemical properties, vermiwash, vermicompost

INTRODUCTION

Growing plants on soil have been developed and practiced from the beginning of human existence. However, this conventional agricultural practice can lead to difficulties in plant growth because nutrient levels in many soil types are unpredictable and may be inaccessible to some plants, also some plant diseases originate from soil. These conventional farming techniques have given the impetus to venture into more reliable alternative methods of growing crops in safer environments to improve yield quantity and quality. It also reduces the dependency on the use of pesticides on crops (Resh and Howard 2012; Treftz and Omaye 2015). Climate change together with irrigated agriculture propels soil salinization, making it more difficult to cultivate glycophytes (plants of non-saline soil). In addition, global population is rapidly increasing and may reach about 9.7 billion by 2050. To sustain this population, the present level of agricultural production has to increase by two folds (UN 2015; Deinlein et al. 2014; Flowers and Muscolo 2015).

Hydroponics is a method of growing plants in water solution which contains the necessary nutrients for plant growth and productivity. Hydroponic agriculture results in higher yields, better quality and enhanced water use efficiency, and can be designed to support production across seasonal variations (Buchanan and Omaye 2013; Gruda 2009; Koyama et al. 2013). In creating hydroponic system, additional elements are required to increase the nutrients including vermicompost and vermiwash. Vermicompost results from bio-oxidative processes in which decomposers disintegrate organic substrates and modify their physical and chemical properties. Earthworms (*Eisenia fetida:* Lumbricidae) were noted for their exemplary ability to breakdown organic matters into rich humus (Domínguez 2004; Ansari and Ismail 2012). Vermiwash is residual liquid that contains excretory and secretory products of earthworms along with micronutrients of the soil that is collected in water having passed through burrows of worm worked soil. It contains essential nutrients that are more readily available to plants and positively influence plant growth and yield (Fathima and Sekar 2014; Jaikishun et al. 2014; Prakash et al. 2016).

Earthworms are abundant in disturbed environments and active in the presence of moisture. They are detritivores and geophages (Ismail, 2005). Their contribution to the soil is evident in the mineralization of nutrients in the form of organic carbon, nitrogen, inorganic phosphorous, potassium, magnesium, and other nutrients in the excreta or worm cast from organic substrates. In making vermicompost, it is beneficial to use appropriate media (e.g. leaves) because it provides the suitable pH to optimize the functionality of earthworms in the soil. Two promising leaves are from lime and neem plants.

Constituents of lime (*Citrus aurantifolia*: Rutaceae) have provided many health benefits to us over the years. Potentially, lime is antibacterial, antifungal, antidiabetic, and anticancerous (Daniels 2006; Balamurugan 2014; Nwankwo et al. 2015). It also contains copious phytochemicals and bioactive compounds that are significantly useful to humans. Compounds extracted from neem (*Azadirachta indica*: Meliaceae) have been used traditionally to cure many infectious diseases and shown to be chemo-preventive with antitumor effects in different types of cancer (Paul et al. 2011; Hao et al. 2014). Neem aids in healthier earthworms because they feed insatiably on neem, converting 7% of the feed into vermicompost daily. Earthworms grow faster and reproduce more rapidly in neem-fed vermireactors (Gajalakshmi and Abbasi 2004).

The objectives of this study are to determine the physicochemical composition of vermiwash and vermicompost produced from different substrates of lime and neem leaves, and to use such vermicompost and vermiwash to grow celery in hydroponic system. Celery (*Apium graveolens:* Apiaceae) has antioxidant potential and many other chemicals that are beneficial to our health (Gauri et al. 2015; Kooti and Daraei 2017).

MATERIALS AND METHODS

Study site

The units were set up at the University of Guyana, Turkeyen Campus while the hydroponics systems were arranged at Georgetown, Prashad Nagar for plants to be grown. Physicochemical analyses were conducted at GuySuCo's laboratory. Microbial analyses were done at the Faculty of Natural Sciences Laboratory, University of Guyana.

Preparing the vermiwash units

Three (3) square five gallon bottles were selected for the units. A tap was fixed on the lower side of each container in such a way to be easily drained and to facilitate collection of vermiwash. About 20 cm of broken pebbles were placed at the bottom of each container followed by 20 cm of coarse sand. Water was allowed to flow through these layers enabling the settling of the basic filter unit. After that, a 22 cm layer of loamy soil was added on top of the filter bed. Then, 25 earthworms were introduced into the soil of containers with 200 g cattle dung + 150 g Lime leaves (T_1), 200 g cattle dung + 150 g Neem leaves (T_2), and 200 g cattle dung + 75 g neem leaves + 75 g lime leaves (T_3) (Figure 1.A). Units were moistened (10 min) and drained every two days. Microbial and physicochemical analyses were conducted on vermiwash and vermicompost. The samples were analyzed for the following physicochemical properties: pH, organic carbon, nitrogen, potassium, available phosphate, exchangeable calcium, exchangeable magnesium, zinc, iron, copper and manganese (Homer 2003).

Preparing the hydroponic system for celery

Small perforations were made at the bottom of small plastic bags (36) that were used for the hydroponic system (Figure 1.B). A mixture of 9 cm of sand and 3 cm paddy straw was placed in each bag. 50 g of vermicompost was added to each bag in rows two (B), four (D) and five (E) from T_1, T_2 and T_3, respectively. The treatment details for the cultivation of celery using hydroponic system are as follows: (i) Treatment A: 3 inches of sand + 1 inch paddy straw + 100 ml of normal nutrients solution per day + 1 celery plant. (ii) Treatment B: 3 inches of sand + 1 inch paddy straw + 100 ml of vermiwash made from T_1 (Lime leaves) per day + 100g of vermicompost from T_1 + 1 celery plant. (iii) Treatment C: 3 inches of sand + 1 inch paddy straw + 100 ml of water per day + 1 celery plant. (iv) Treatment D : 3 inches of sand + 1 inch paddy straw + 100 ml of vermiwash made from T_2 (Neem leaves) per day + 100g of vermicompost from T_2 + 1 celery plant. (v) Treatment E : 3 inches of sand + 1 inch paddy straw + 100 ml of vermiwash made from T_3 (equal proportions of neem and lime leaves) per day) + 100g of vermicompost from T_3 + 1 celery plant.

Figure 1. A. Earthworms used in the study; B. Arrangement of seedlings

Microbial analysis (Aneja 1996)

Preparation of nutrient agar

About 14 g of nutrient agar powder was weighed and suspended in 500 ml of distilled water in a 1000 ml flask. The mixture was then boiled and swirled. After dissolving, the mixture was then placed in an autoclave at 121^0C for sterilization for about 20 minutes then cooled to 47-50^0C. It was then poured into sterile Petri dishes allowed to gel.

Culturing of microorganisms

About 0.1 g of soil sample was weighed and dissolved in 10 ml of water in a test tube. Each Petri plate was labeled according to the respective substrate that was streaked onto it. After streaking, the plates were sealed and incubated at 37ºC for 24 hours. Total microbial count (included bacteria and fungi) was done after incubation along with morphological description of each colony present.

Gram staining procedure for identifying the type of bacteria

The chosen bacterial colony from the cultured plate was removed with an inoculating loop and thin smear of the isolated bacterial colony was made on the slide in a circular direction on a drop of distilled water. The smear was then fixed by swiftly heating through a Bunsen flame. After drying, the slide was then flooded with crystal violet for 30 seconds and then washed with distilled water. After washing off crystal violet from the side, the slide was then flooded with Gram iodine for 1 minute and then decolorized by tilting the slide and dropwise rinsing with 95% ethanol. Slide was then washed with distilled water for few seconds and stained with 5-6 drops of safranin for 20 seconds then rewashed. After counterstaining, the slide was blotted and air-dried. It was then examined under a microscope using oil immersion to identify bacterial colonies.

Neem treatment and pest management

About 600 g of neem leaves were collected and boiled with 1l of water. After boiling, the mixture was diluted with 5l of water and mixed with 50 ml of soap. The neem extracts were then sprayed as pest management on plants three weeks after planting in treatments B, D and E.

Plant growth parameters

Plant growth parameters such as plant height and number of leaves recorded per week for period of 6 weeks. Percentage increase was calculated by using the following formula:

% increase in plant height /number of leaves = (week 6-week 1)/week 6 x 100

Agronomic data taken at harvesting included height, root biomasses, number of shoots, number of leaves, and diameter of stem.

RESULTS AND DISCUSSION

Vermicompost made from leaves of neem (T_2) and lime+neem (T_3) was slightly acidic (pH 5.89) while it was more neutral for vermicompost made from T_1 (pH 7.54). All pH levels except T_2 were within the pH ranges 6.5-7.5 which is the pH that most plant nutrients are optimally available for plant growth. Nitrogen level was the lowest in T_3 followed by T_1 and was the highest in T_2. Statistical analysis (ANOVA) showed that the difference in physicochemical parameters is not significant ($p=0.34$). Nitrogen is required by plants in the largest quantity and is most frequently the limiting factor in growth, development and productivity. A plant receiving adequate nitrogen will exhibit enhanced growth (Perchlik and Tegeder 2017). Phosphorus was the lowest in T_3 followed by T_1 and the highest in T_2. Potassium level was lowest in T_1 followed by T_3 and the highest in T_2. Potassium is an essential plant nutrient and is required in large amounts for proper growth and reproduction of plants. Copper was the lowest in T_2 followed by T_3 and the highest in T_1. Copper activates the biosynthesis of enzymes and proteins critical for the metabolisms in plants (Yruela 2005). Zinc was the lowest in T_2 followed by T_1 and was the highest in T_3 (Table 1).

Zinc is a plant micro-nutrient and is essential for the synthesis of enzymes essential for nitrogen metabolism, energy transfer and protein synthesis. Therefore, its deficiency will result in significant growth retardation and reduction in plant yield (Hafeez et al. 2013). Manganese was the lowest in T_3 followed by T_2 and the highest in T_1. Manganese contributes significantly to plant physiological systems and structural formation of proteins designed for photosynthesis and other biochemical reactions (Millaleo et al. 2010). Iron was the lowest in T_2 followed by T_3 and the highest in T_3. Iron is needed for chlorophyll synthesis and maintenance of chloroplast structure. Deficiency in iron leads to poor plant health, reduced productivity and quality of yield (Rout and Sahoo 2015). Magnesium was the lowest in T_3 and the highest in T_1. Magnesium is an essential macronutrient for plants. It has a range of key roles in many plant physiological functions (Guo et al. 2005). Calcium was the lowest in T_2 and the highest in T_1. Calcium is considered a secondary plant nutrient and is required for structural function in cell walls and membranes (Marschner 1995).

Microbial analyses of vermiwash and vermicompost were conducted during the third week to ascertain the presence of microbes. Total microbial count on vermiwash was the highest in T_1 and the lowest in T_3. The highest number of bacteria was seen in T_1 as well as the highest number of fungi as compared to the other samples. The highest microbial count in T_1 is due to the substrate used to prepare the vermiwash which is lime leaves which may be attributed to weak presence of antimicrobial properties. Also, microbes were deposited from earthworm casting and some also came from the cattle dung used, however, earthworm casting and cattle dung was also used to prepare the other vermiwash units. The main reason for the highest amount of microbes in T_1 is the use of lime leaves compared to neem leaves and a combination of neem and

lime leaves in the other two vermiwash units respectively because of the presence of several antimicrobial active ingredients in neem leaves. In vermicompost, similar results were obtained where the highest number of bacteria, as well as fungi, was found in vermicompost from T_1 (Figure 2). Statistical analysis (ANOVA) done for results obtained on vermiwash showed that the results were not statistically significant. Statistical analysis (ANOVA) showed that the *p*-value 0.17 ($p > 0.05$) and for treatments and *p* of 0.44 ($p > 0.05$) for the different microbes. For statistical analysis conducted on vermicompost, it was seen that results were statistically significant for the different microbes and were not statistically significant for treatments where treatments have a p-value of 0.48 ($p > 0.05$) and the different microbes have a p-value of 0.01 ($p < 0.05$). Earthworm activities enhance the beneficial population and in some cases may have repressive responses to pathogens in the soil which allows for healthier plants (Yakushev 2011; Kiyasudeen et al. 2016).

Plant heights were measured on a weekly basis for 6 weeks and there was a significant difference between the plants' height recorded over the 6 weeks period (based on ANOVA). The highest mean growth and percentage increase (80%) were seen in plants grown in D treated with T2 followed by plants grown in B treated with T1, while the lowest height was seen in plants grown in the control medium (C)which was treated with only water (Table 2). Analyses showed the highest level of nitrogen present in T2 followed by T1 and nitrogen is a significant player of chlorophyll and boosts plant growth and development (Perchlik and Tegeder 2017).

Number of leaves was counted on a weekly basis for 6 weeks and a significant difference was also in the number of leaves over the 6 weeks period. The highest number of leaves was found in plants grown in B treated with T1 followed by those grown in D treated with T2 and the lowest number of leaves was found in the control. Magnesium was highest in T1 followed by T2 (Table 3). Magnesium plays a pronounced role in many enzyme activities and structural stabilization of tissues and its deficiency can result in poor plant health (Guo *et al.* 2016). Magnesium deficiency is reflected in diminutive roots and shoots and leaves with necrotic spots resulting from the impairment of carbon metabolism and chlorophyll deficiency (Brady et al. 2005; Guo et al. 2005; Herman and Verbruggen 2005).

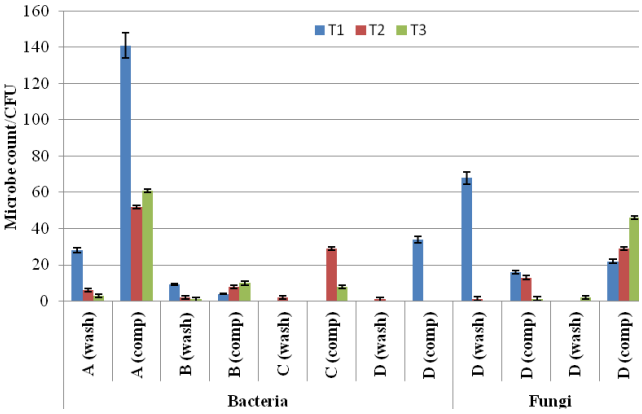

Figure 1. Microbial count of vermiwash and vermicompost (Mean ±SD)

Table 1. Nutrient status of vermicompost produced from various plant materials

Treatment	Physico-chemical parameters (mg/kg)										
	pH	N	P	K	Cu	Zn	Mn	Fe	Mg	Ca	OC
T_1	7.54	13625	123	28450	0.65	38.1	39.8	4.92	239	940	12.6
T_2	5.89	14725	183	50700	0.15	31.0	23.2	1.49	210	478	29.2
T_3	6.82	11175	99.8	35850	0.28	41.4	15.9	2.50	156	503	29.5

Table 2. Plant height obtained from celery plants grown in six different treatments (Mean±SD)

Treatment	Week						% increase
	1	2	3	4	5	6	
A	5.57±1.83	10.18±3.99	12.57±3.63	16.08±5.5.54	16.85±8.68	18.27±9.45	69.51
B	6.12±2.13	10.18±2.94	12.45±1.61	17.92±2.52	21.42±3.75	24.03±3.81	74.53
C	4.32±0.32	8.97±1.03	10.12±1.41	12.28±1.86	12.82±1.99	13.52±2.55	68.05
D	5.17±0.96	11.58±3.52	12.4±2.60	18.78±4.30	23.67±6.03	26.1±6.35	80.19
E	5.48±0.33	10.63±5.54	10.7±4.78	15.55±7.51	18.18±8.41`	20.12±8.71	72.76

Table 3. Average leaf number obtained from celery plants (Mean ±SD).

Treatment	Week-number of leaves						% increase
	1	2	3	4	5	6	
A	7±2.28	7±2.28	11.83±4.12	13.5±6.77	15.83±8.08	16.33±8.26	57.13
B	8±1.26	8±1.26	13.33±4.59	19.33±4.84	22.33±4.55	23.83±4.45	66.43
C	8.17±1.33	8.17±1.33	10.33±3.44	11.5±2.26	13.33±2.50	17.33±4.32	52.86
D	7±3.10	7±3.10	11.83±6.11	18.67±4.27	21.83±4.53	24.33±2.73	71.23
E	6.67±2.16	6.67±2.16	10.83±4.62	15.5±6.68	18.67±8.07	20.33±8.78	67.19

Table 4. Agronomic features after harvest

Treatment	Height/cm	No. of shoots	No. of leaves	Diameter of main stem/cm	Root weight harvest/g
A	14.8±5.6	3±1.2	13±3.6	0.3±0.01	3.1±1.2
B	17.2±8.2	5±3.4	24±5.7	0.4±0.02	19.2±5.4
C	11.5±4.7	4±2.8	12±6.2	0.2±0.01	3.9±2.4
D	18.5±6.7	5±3.1	17±9.1	0.4±0.01	28.8±8.7
E	15.0±5.8	4±2.1	14±5.6	0.4±0.00	20.1±6.5

Overall plants parameters showed that plants that were grown in treatments D and B showed more pronounced growth followed by those in E then F and A and control C. Highest plant height was seen in those that were grown in D due to the high amount of nitrogen present in the vermicompost. Number of leaves was significantly high in B as compared to the others. Width of the main stem was equal in B, D and E, while root weight after harvest was significantly higher in D as compared to the others (Table 4). Statistical analysis (ANOVA) done on the overall parameters indicated a statistical significance for the different parameters with *p*-value of 2E-05 whereas it was statistically not significant for treatments with a *p*-value of 0.047 ($p > 0.05$).

In conclusion, vermiwash and vermicompost when used as a composite can supplant nutrient solution in hydroponic systems since it promotes greater plant growth in its entirety. Vermiwash and vermicompost made from lime leaves have proven to be a better substitute for plant growth resulting in taller plants with greater width. They constitute adequate amount of macro and micronutrients for plants and release these nutrients slowly so that they are readily accessible and remain in the soil much longer. Also, these nutrients are in right balance, hence they don't react to affect the quantity available or absorption of others and thus maintaining a healthy soil ecosystem.

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Amino acids profile and protein functional properties of *Chrozophora oblongifolia* seeds from Kordofan Region, Sudan

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Abstract. *Abdalgader SIA, Ahmed AI. 2019. Amino acids profile and protein functional properties of* Chrozophora oblongifolia *seeds from Kordofan Region, Sudan. Trop Drylands 3: 17-21.* Species of Chrozophora genus (Euphorbiaceae family) have been traditionally used as food with many of these species showing high content of protein and oil with high percentages of fatty acids. The present research was conducted to study the amino acids profile and protein functional properties of *Chrozophora oblongifolia* seeds. The fresh seeds samples were obtained from North and West Kordofan regions, Sudan. The protein functional properties, i.e., water absorption capacity, oil absorption capacities, emulsifying capacity, foaming stability, bulk density and crude protein were determined, then the amino acids profile was investigated using an amino acids analyzer (L-8900 Hitachi-Hitech, Tokyo, Japan) under the experimental conditions recommended for protein hydrolysates. There were seven essential amino acids namely lysine, histidine, threonine, methionine, valine, isoleucine and phenylalanine, and nine non-essential amino acids namely arginine, aspartic acid, serine, glutamic acid, glycine, proline, alanine, cysteine and tyrosine. Glycine with a range of 0.99-1.02 g/100g proteins, while arginine with a range of 8.43-8.87 g/100g protein was the highest. Leucine and isoleucine, which were limiting amino acids in most foodstuffs, were in ranges of 7.27-7.59g/100g and 5.24-56 4.19 g/100g, respectively. Statistical analysis of the seeds protein concentrates showed that significant differences ($p \leq 0.05$) in crude protein and water absorption capacities, oil absorption capacities, foaming stability and bulk density were found between the two different collection regions, while there were no significant ($p \leq 0.05$) differences found in emulsifying capacity and foaming capacity. The protein concentrates indicated higher protein content for North Kordofan seeds (83.33%) than that of 80.6% for West Kordofan seeds. This study concluded that *C. oblongifolia* seeds can be considered as a cheap source of edible protein which had rich of essential amino acids

Keywords: Amino acids profile, functional properties, *Chrozophora oblongifolia* seeds, Kordofan region, Sudan

INTRODUCTION

Plants have been used for thousands of years to flavor and conserve food, treat health disorders and prevent diseases. Species of *Chrozophora* genus (Euphorbiaceae family) are distributed in West Africa and Asia and they are monoecious, shrubby herbs and annual plants. Their leaves, stems and fruits have been used in food and traditional medicine for the treatments of infectious diseases; many of these species showed high content of protein and oil with high percentages of fatty acids (Ahmed et al. 2014). The biological activity of the *Chrozophora* plants received increased attention to discover new leading compounds for treatment of diverse ailments (Galal and Adam 1988).

Methanol extracted from the parts of *Chrozophora oblongifolia* showed highest antioxidant and hepatoprotective activities, and it had a valuable biological source of drugs that enhances fertility (Kamel et al. 2018). In 1995, a study was conducted at Safiola company-Sudan to screen the seeds oil characteristics. Results showed that it has 0.29% moisture, 2.7% free fatty acids, 1.9 mq/kg peroxide value, 102.9g/100g iodine value close to cottonseed and rapeseed oils. Also, the trace metals contents were within the normal limit, and its refining and bleaching behaviors were very similar to other normal vegetable oils (Galal and Adam 1988).

Thus, the industrial potentiality of *Chrozophora* oil should be explored. Therefore, exploitation of *Chrozophora* seed as alternative source of proteins and oil need to be investigated. The aim of present work was to analyze amino acids profile and investigate protein functional properties of seeds of *C. oblongifolia* plant.

MATERIALS AND METHODS

Plant materials

The fresh seeds of *C. oblongifolia* were collected from two different locations from West and North Kordofan States, Sudan. The plant materials were air-dried in the laboratory and then ground into powder form using a mortar, shieved, and then stored in air-tight bottles pending the analyses.

Amino acids analysis

The amino acid content (except for tryptophan) in the seeds of *C. oblongifolia* plant was determined using an amino acid analyzer (L-8900 Hitachi-Hitech, Tokyo, Japan) under the experimental conditions recommended for protein hydrolyzates. Samples containing 5.0 mg of protein were acid hydrolyzed with 1.0 ml of 6 N HCl in vacuum-

sealed hydrolysis vials at 110°C for 22 h. Ninhydrin was added to the HCl as an internal standard. The tubes were cooled after hydrolysis, opened and placed in a desiccator containing NaOH pellets under vacuum conditions until dry (5-6 days). The residue was then dissolved in a suitable volume of NaS buffer, pH 2.2 filtered through a Millipore membrane (0.22-lm pore size, Millipore, Billerica, MA, USA) and analyzed for amino acids by ion-exchange chromatography in a Beckman (model 7300, Pickering Laboratories, Inc. Mountain View, CA, USA) instrument, equipped with an automatic integrator. Amino acid nitrogen was determined by multiplying the concentration of individual amino acids by corresponding factors calculated from the percentage N of each amino acid (Mosse 1990). The ammonia content was included in the calculation of protein nitrogen retrieval, as it comes from the degradation of some amino acids during acid hydrolysis (Yeoh and Truong 1996; AOCS 1993). The ammonia nitrogen content was calculated by multiplying the ammonia content by 0.824 (N = 82.4% NH_3).

Amino acids calculation

The amount of amino acid obtained was calculated (g/100g) by the formula:

$$X = \frac{\text{Area of Asp in the sample X Area of internal std (AABA) X amount of std X dilution factor}}{\text{Area of Asp std X Area of sample internal std X sample weight}}$$

Where:

X = represents the amount of amino acid (g/100g)

Preparation of *Chrozophora oblongifolia* seeds protein concentrate

Protein was extracted from *C. oblongifolia* protein concentrate using alkali solution with isoelectric precipitation and freeze-drying. The dried defatted seeds were weighed and suspended in distilled water in 1:10 (w/v) ratio using magnetic stirrer, the mixture was stirred for 1 hour, while adjusting the pH at 9.0 using sodium hydroxide NaOH solutions (4M). Then, the mixture was centrifuged at 3500 rpm for 15 minutes at ambient temperature. The supernatant was transferred into a beaker and stirred for another 30 minutes and the pH was adjusted to 4.5. The supernatant was left undisturbed for cold precipitation overnight in 4°C freezers. After that, the supernatant was carefully siphoned off and the protein slurry was washed 3 times with distilled water by adding distilled water and centrifuging at 3500rpm for 10 minutes at 4°C. The pellets were then mixed together and some distilled water was added in there. The pH was adjusted at 7.0. The slurry was kept overnight inside -80°C freezer before it was freeze-dried (Chandi and Sogi, 2007). The sample inside the freeze dryer took 2 to 3 days before it was completely dried. The protein concentrates obtained from seeds were weighed using analytical balance.

Crude protein content

The crude protein content was determined in protein concentrate of *C. oblongifolia* plant seeds by macro-Kjeldahl method according to the official methods analysis (AOAC 2005).

Functional properties of protein concentrate

Water absorption capacity: The water absorption capacity was determined according to method described by Jyothirmayi et al. *(*2006) as follows: Only 0.1 g of *C. oblongifolia* protein concentrate was taken from the sample mixed with 1 ml of distilled water. The slurry was centrifuged at 3000 rpm for 15 minutes. The supernatant was removed, then the pellets were drained for 30 minutes and the gain weight per unit weight was reported as water absorption capacity (g/g).

Oil absorption capacity: One gram of *C. oblongifolia* protein concentrate was mixed with 10 ml of refined sunflower oil, vortex thoroughly, and centrifuged at 3000 rpm for 15 minutes. The oil absorbed by the samples was noted and expressed as oil absorption capacity (g/g) (Beuchat 1977).

Foaming capacity and foam stability: Foaming capacity of *C. oblongifolia* protein concentration was determined by measuring the volume of foam immediately after the introduction of air (90 cm^3/min) for 15 seconds into 5ml of 0.2% protein solution in 0.05M phosphate buffer (pH 7.4) (Kato 1989). Foam stability was calculated using the following equation:

$$FS = V0\ (\Delta t/\Delta V)$$

Where F: foaming, S: stability, ΔV: the change in the volume of foam (V), occurring during the time interval, Δt (30 min), and V0 is the volume of foam at 0 time.

Emulsifying capacity: For the determination of emulsifying capacity, 50 g of protein suspension were transferred into a blender vat and the sunflower oil was added, under continuous mixing until the emulsion was destroyed (Beuchat, 1977). Measurements were performed at 22 ± 1°C and the emulsifying capacity was expressed as ml of oil used for the emulsification of 1 g of *C. oblongifolia* protein derivatives.

Bulk density: The bulk density was determined according to the methods outlined by Okaka and Potter (1977). Ten grams of protein isolate were put into 100 mL measuring cylinder, then tapped several times on the laboratory bench till the isolate stopped settling, the values were expressed as g/cm^3.

RESULTS AND DISCUSSION

Amino acids profile

Most of the amino acids were found to be presence in *C. oblongifolia* seeds samples investigated except tryptophan (Table 1). A total of 17 amino acids consisting of eight essential, namely lysine, histidine, threonine, methionine, valine, isoleucine, leucine, and phenylalanine and non-essential amino acids namely arginine, aspartic acid, serine, glutamic acid, glycine, proline, alanine, cysteine and tyrosine are presented in Table 1. The highest

value of essential amino acids found in North Kordofan region seeds was leucine, isoleucine and valine, while the highest value of essential amino acids of *C. oblongifolia* seeds in West Kordofan was leucine, isoleucine, valine and threonine. On the other hand, the lowest values of essential amino acids from both samples were histidine and methionine. Non-essential amino acids arginine, alanine and aspartic acid from *C. oblongifolia* seeds in North and West Kordofan had the highest value compared to the lowest values of serine, glycine, glutamic acid, tyrosine, and cysteine from both samples' regions. The amino acid with the least concentration was glycine with a range of 0.99-1.02 g/100 g proteins, while arginine with a range of 8.43-8.87 g/100g protein was the highest (Table 1).

The ranges of essential amino acids obtained from the different locations of *C. oblongifolia* seeds samples in present study were in agreement with *Chrozophora brocchiana* seeds amino acids reported by Ahmed (2014) and also was within the same range for reference pattern protein by FAO (1981) standards which were indicated for leucine and isoleucine. These were limiting amino acids in most feedstuffs that were presently ranging from 7.03g/100g protein for leucine and 4.19 g/100g protein for isoleucine in those mentioned in the FAO standards. The value of essential amino acids concentration obtained in this study was in line with Ahmed (2014) who showed that *C. oblongifolia* had higher protein contents of essential amino acids such as leucine, isoleucine, and valine. The results of this study showed that the protein of *C. oblongifolia* seeds had higher quantity of amino acids such as arginine, alanine leucine, aspartic acid, isoleucine, and valine.

Table 1. Amino acids profile (g/100g crude protein) of *C. oblongifolia* seeds

Amino acid	Samples source	
	North Kordofan	West Kordofan
Essential		
Lysine	2.31	3.23
Histidine	1.80	1.16
Threonine	3.16	3.87
Valine	4.13	5.33
Methionine	1.02	1.91
Isoleucine	5.24	5.56
Leucine	7.27	7.59
Phenylalanine	3.12	3.57
Non-essential		
Aspartic Acid	6.11	6.74
Alanine	8.10	7.23
Serine	1.46	1.03
Tyrosine	1.09	1.04
Glutamic Acid	1.51	1.75
Glycine	1.02	0.99
Ammonia	6.21	6.32
Arginine	8.43	8.87
Cysteine	1.65	1.55
Proline	3.02	2.97

Protein content of the concentrates

Protein contents in that protein concentrate had a high amount in both North and West Kordofan regions which were 83.33 and 80.66%, respectively (Table 2). The variations in protein contents of different protein isolates could possibly be due to extent of soluble proteins present in raw materials. The variations in protein contents are attributed to genetic makeup of the sources of proteins along with some environmental factors. The proteins known as polymers of amino acids and their relatives' proportion represent its quality that is dependent on genetic makeup of sources of proteins such as (legumes). This result is similar to some legumes such as pea protein concentrate of 83.61% and pigeon pea of 82.92% which were reported by the above mentioned authors (Masood and Rizwana 2010).

Functional properties

Water and oil absorption capacity

Seeds protein concentrates obtained from North Kordofan seeds exhibited water absorption capacity of 2.9 g/g, and West Kordofan seeds protein was absorbed 2.26 g/g (Table 2). However, these values were higher than 1.45 for peanut powder reported by Monteiro and Prakash (1994). On the other hand, this value is in agreement with the 2.62 g/g in mung bean protein concentrates revealed by Du et al. (2018). Therefore, the protein concentrates obtained from defatted *C. oblongifolia* seeds had low water absorption, but are still considered high in the range of water absorption capacity of glutinous food, while the oil absorption capacity of protein concentrate was 3.73 g/g for North Kordofan seed oil and 3.13 g/g for West Kordofan seeds oil. The low value of oil absorption may be attributed to the presence of large proportion of hydrophilic groups and polar amino acids on the surface of the protein molecules (Sathe et al. 1982). Protein has both hydrophilic and hydrophobic properties, thereby can interact with water and oil in food. The varied values of WAC might be due to the protein structure and amount of polar amino acids, whereas the OAC difference might be due to the difference in nonpolar side chains binding the oil (Yi-Shen 2018).

Emulsion absorption capacity

The emulsion absorption, for both samples, revealed almost a similar absorption rate of 3.33 g/g for North Kordofan protein concentrates and 3.00 g/g for West Kordofan protein concentrates. Results are comparable to the earlier findings of Bugis (2009) who reported a value of 1.32 g/g emulsion absorption for lupine protein isolates. These findings are in line with Makri et al. (2005) who stated that protein being the surface-active agent can form and stabilize the emulsion by creating electrostatic repulsion on oil droplet surface.

Table 2. Protein functional properties of *Chrozophora oblongifolia* seeds

Parameter	North Kordofan	West Kordofan
Protein content %	(83.33)a ±1.52	(80.66)b ±1.52
Water absorption capacity g/g	(2.90) a ±0.10	(2.26)b ±0.25
Oil absorption capacity g/g	(3.73)a ±0.25	(3.13)b ±0.15
Emulsion absorption g/g	(3.33)a ±0.25	(3.00)a ±0.10
Foaming capacity %	(21.66)a ±0.57	(20.00)a ±1.00
Foaming stability g/ml	(2.33)b ±1.52	(2.43)a ±1.52
Bulk density g/cm3	(0.73)a ±0.04	(0.56)b ±0.05

Note: *All Determinations were carried out in triplicate and mean value ± standard deviations (SD). Means not sharing superscript letters in a column were significant different

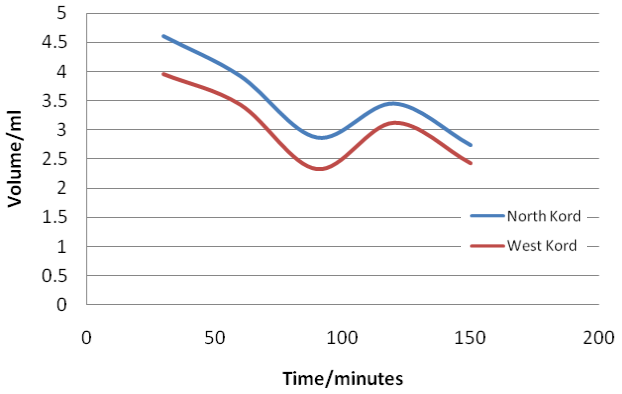

Figure 1. Foaming stability of protein concentrate of *Chrozophora oblongifolia* seeds

Foaming capacity and stability

Foaming capacity of protein concentrates of North Kordofan and West Kordofan seeds was 21.66% and 20%, respectively, where it reached a maximum at pH 9. The foaming capacity of both locations of protein concentrates was affected by pH and it tended to decrease as pH decreased. The same trends were found in foaming stability. The foaming stability of North Kordofan protein was reported at a time of 30, 60, 90, 120 and 150 minutes were 4.60, 3.92, 3.43, 2.87, 2.33 g/ml, respectively, for the same mentioned times the West Kordofan protein was 3.95, 3.45, 3.12, 2.74 and 2.43g/ml, respectively (Figure 1). The foaming capacity of *C. oblongifolia* protein concentrates in present investigation was found to be lower value when compared with the results of the commercial protein for albumin studied by Moharram et al. (1984), while the foaming capacity declined due to several factors, including the source and composition of protein as well as the temperature and the solubility (Jasim 1983).

Bulk density

The bulk density of protein concentrates from two different regions of North Kordofan and West Kordofan seeds were found to be 0.73 g/cm^3 for North Kordofan and 0.56 g/cm^3 for West Kordofan (Table 2). These results were in agreement with Masood and Rizwana (2010) who reported a value of 0.71 g/cm^3 for bulk density of legumes protein isolates.

In conclusion, the amino acid with the lowest concentration was glycine, while arginine had the highest value found in the two different production regions. The range of essential amino acids obtained in this study was within the same range for reference pattern protein required by FAO standards especially leucine and isoleucine which were limiting amino acids in most feedstuffs. This study investigated the nutritional value of *C. oblongifolia* seeds as a cheap source of edible protein which had rich in essential amino acids. Characterization of the properties of protein concentrates obtained from *C. oblongifolia* seeds is expected to improve the industrial application of crude protein substances. More studies to elaborate the use of this protein mixed with other food materials and their effects on consumer acceptability are needed in future work as recommended point of view.

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Levels of anthocyanin, βeta carotene and antioxidant activity of functional biscuits flour of purple, yellow and white fleshed sweet potatoes

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Abstract. *Abidin Z, Jutomo L, Harini TS. 2019. Levels of anthocyanin, βeta carotene and antioxidant activity of functional biscuits flour of purple, yellow and white-fleshed sweet potatoes. Trop Drylands 3: 22-28.* Flour produced from sweet potato has the potentials to partly substitute wheat flour, which is relatively expensive in tropical regions such as Indonesia. This study aimed to determine the levels of anthocyanin, beta carotene and antioxidant activity of functional biscuit formulas based on flour of purple, yellow and white sweet potatoes from Sumba Barat Daya District, East Nusa Tenggara Province. The purple, yellow and white sweet potatoes were processed into flour then cooked into biscuits with varying mixing ingredients. The levels of anthocyanin, beta carotene and antioxidant activity were analyzed using UV-Visible Spectrophotometry. The results showed that the highest levels of biscuit anthocyanin (95.05 ppm) was found in 75% purple sweet potato flour + 25% wheat flour formula. The highest beta carotene level of biscuit (10,190.44 µg/100 g) was found in 75% of yellow sweet potato flour + 25% flour formula. In biscuit products, the highest antioxidant activity (61.57%) was found in the treatment of 75% purple sweet potato flour + 25% flour formula.

Keywords: Anthocyanin, antioxidant activity, beta carotene, biscuits

INTRODUCTION

Biscuits are well-known baked food products with good taste, sweet and crunchy texture. Biscuits are usually made from wheat flour, sugar and margarine. Yet, wheat flour is quite expensive in price, particularly in regions where wheat cultivation is not common such as in the wet tropics. Thus, substitute flour produced from raw materials that are locally grown is explored which can substitute wheat flour up to 50-75%. One material that has the potential is sweet potato flour. Processed food products utilizing sweet potato flour as a partial substitute of wheat flour have no significant differences in chemical, physical and organoleptic properties as compared to those of wheat flour. Sweet potato flour can also be developed as a functional food.

Awareness of the importance of functional food has started to rise in early 1980, when many people of the world began to emphasize the choice of foods that can prevent health problems in the elderly and diseases related to lifestyles, such as diabetes, heart disease, high blood pressure, hypercholesterolemia, allergies, infectious diseases, and cancer. Food experts intensely promoted the third function of food, in addition to being a source of nutrition and enjoyment. This tertiary function modulates physiological control, known as 'biological regulatory function', in the immune system, glands, nerves, blood circulation, and digestion. The food must contain compounds that can activate this ability. These compounds are called 'functional factors'. Foods that contain a variety of functional factors and are processed for the purpose of providing a specific impact on health care are called 'functional foods' (Arai 2002).

Functional food is food that uses specific ingredients in its formulation because of its properties; hence, it contains several functional factors, among others is, antioxidant property. An antioxidant can remove free radicals that threaten healthy cells in the human body, leading to the development of cancer cells and endangering human health or causing degenerative diseases, which have not been well cured at the moment (Pietta 2000; Mukherjee and Mitra 2009).

Flour produced from purple, yellow and white sweet potatoes exhibits high antioxidant content as compared to other flour products. The antioxidants include anthocyanin, β-carotene, and other polyphenol compounds. Anthocyanin is known to be one of the groups of natural products found in plants that can act as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants, allergy, antiviral, and anti-inflammatory (Pietta 2000). Purple-fleshed sweet potato contains anthocyanin around ± 519 mg/100 g weight (Kumalaningsih 2006). Yellow and white sweet potatoes contain Vitamin A of, respectively, 900 IS, and 7700 IS in the form of β-carotene. β-carotene is one of the carotenoids that can produce a higher amount of provitamin A than other types of carotenoids. Provitamin A is very easily absorbed by the digestive tract of the human gastrointestinal system which is then converted into vitamin A.

Inappropriate implementation of sequestration technology and processing of functional food products as well as packaging and storage of the products can lead to reduced quantity and quality of antioxidants found in sweet potatoes. Processing of purple sweet potato will affect the anthocyanin level; for instance, 10-30% of anthocyanin damage did occur in the Ayamurasaki purple sweet potato varieties as a result of frying and steaming (Widjanarko 2008).

This study aimed to determine the levels of anthocyanin, beta carotene and antioxidant activity of functional biscuit formulas based on flour of purple, yellow and white sweet potatoes grown in Sumba Barat Daya District, East Nusa Tenggara Province, Indonesia.

MATERIALS AND METHODS

Fresh purple, yellow and white sweet potatoes were taken from Tena Teke Village, Wewewa Selatan Sub-district, Sumba Barat Daya District, East Nusa Tenggara, Indonesia. Production of sweet potato flour and biscuit was carried out at Agricultural Post Harvest and Processing Laboratory, Faculty of Agriculture, Universitas Nusa Cendana, Kupang, Indonesia. Meanwhile, the chemical analysis of biscuits was carried out at the Pratama Chem-Mix Laboratory, Yogyakarta, Indonesia.

The production process of purple, yellow and white sweet potato flour

Sweet potato tubers collected in Sumba Barat Daya District were processed into sweet potato flour following the stages as presented in the flow-chart in Figure 1 while ingredients used in the production of biscuits are listed in Table 1.

Steps of production processes of biscuits from sweet potato flour are presented in Figure 2. Mixing of raw materials was carried out using the following steps: (i) The margarine was mixed with fine sugar using a mixer for two minutes until it turns white, (ii) Egg yolk was poured and mixed thoroughly with the above mixture (step 1), (iii) The flour was slowly poured and mixed with other materials then the mixture was squeeze

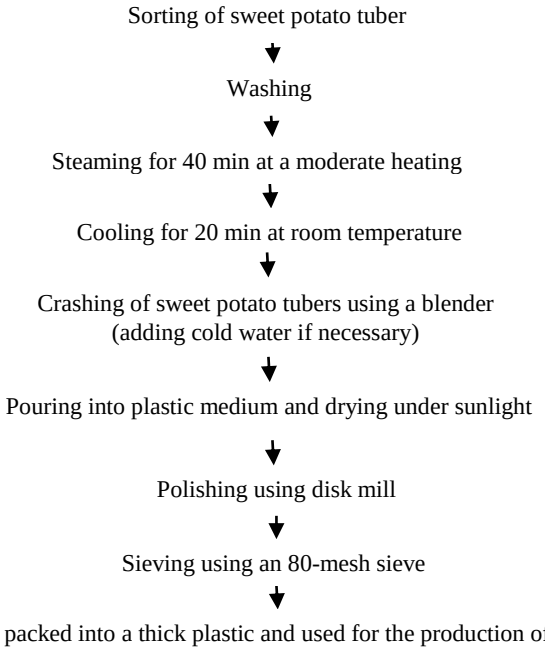

Figure 1. Flowchart of sweet potato flour production

Table 1. Formulation of ingredients used to produce biscuits from sweet potato flour

Ingredient	Treatment combination					
	A1 (pspf)	A2 (pspf)	B1 (yspf)	B2 (yspf)	C1 (wspf)	C2 (wspf)
Sweet potato flour	50% (100 g)	75% (150 g)	50% (100 g)	75% (150 g)	50%(100 g)	75%(150 g)
Wheat flour	50% (100g)	25% (50g)	50% (100g)	25% (50 g)	50%(100g)	25% (50 g)
Eggs	60 g	60 g	60 g	60 g	60 g	60 g
Fine sugar	60 g	60 g	60 g	60 g	60 g	60 g
Margarine	50 g	50 g	50 g	50 g	50 g	50 g
Milk powder	20 g	20 g	20 g	20 g	20 g	20 g
Baking powder	1 g	1 g	1 g	1 g	1 g	1 g

Note: pspf = purple sweet potato flour, yspf = yellow sweet potato flour, and wspf = white sweet potato flour

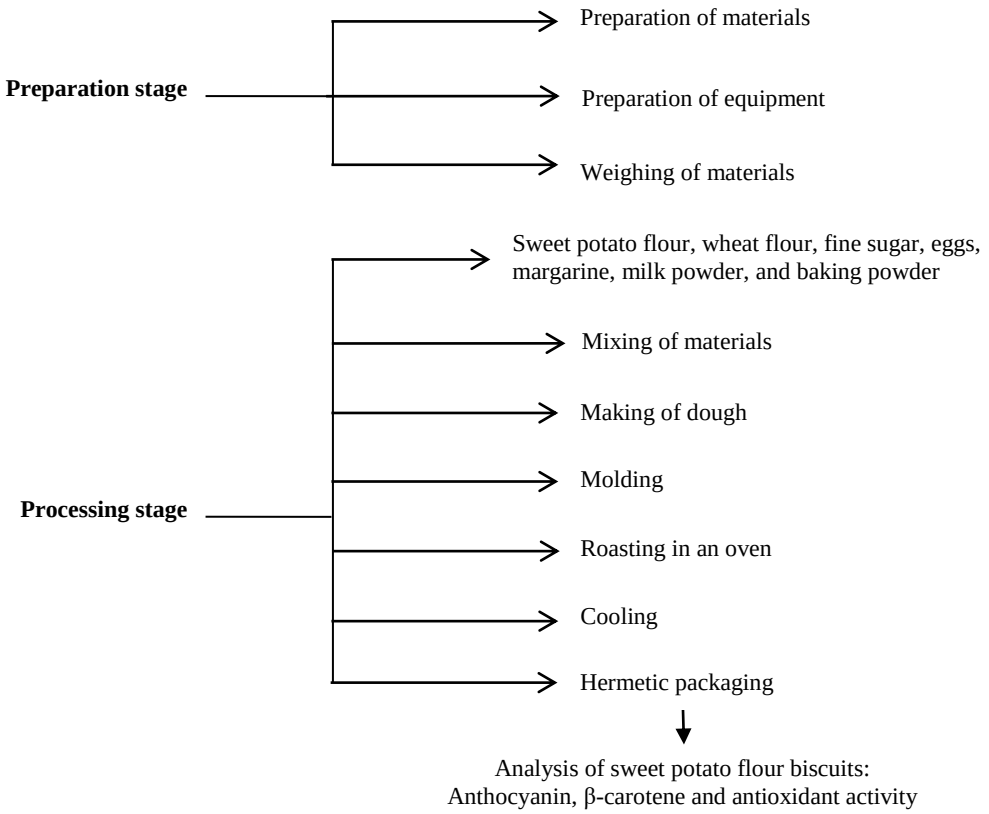

Figure 2. Flowchart of production processes of biscuits from sweet potato flour

well to produce elastic dough, (iv) The dough was flattened by using a wood roll tool to the same thickness. The dough was then printed using a biscuit mold and a hole was put on the top of the mold using a fork. The dough was then roasted in moderate heating for 10 minutes, (v) The biscuits were taken out from the oven and then cooled first to release the hot steam prior to packaging, (vi) Packaging: cooled biscuits were immediately packed using small stops or thick plastic to keep them remain crispy for an extended time and to maintain long-term shape.

Analysis of anthocyanin content and antioxidant activity

Extraction

Extraction of the sweet potato flour was based on the method developed by Lee et al. (1998). Five grams of sweet potato flour were extracted five times each using 10 mL of 15% acetic acid in methanol for 30 minutes. The extraction was carried out using a shaker. The combined extract was then centrifuged at 12000 x g for 10 minutes at room temperature. The supernatant was evaporated under vacuum at 40°C to make it dry. This dry extract was then dissolved in 25 mL methanol for further analysis.

Total anthocyanin measurement

Total anthocyanin measurement of the sweet potato flour was based on the method developed by Prior et al. (1998) and Giusti and Wroistad (2000): 0.05 mL sample was put into 2 test tubes. The first test tube was added with 4.95 mL potassium chloride (0.025 M) buffer (pH 1.0) and the second test tube was added with 4.95 mL sodium acetate (0.4 M) buffer (pH 4.5). The pH of potassium chloride and sodium acetate buffers was adjusted using concentrated HCl. The two buffer solutions were kept at room temperature for 15 minutes, and the absorbance of the two was measured by using a spectrophotometer at a wavelength of 520 nm and 700 nm. The absorbance value was calculated using the formula A = (A520-A700) pH 1-(A520-A700) pH 4.5. Anthocyanin concentration was calculated as cyanidin-3-glycoside using a molar extraction coefficient of 29 600 L/cm and a molecular weight of 448.8. Anthocyanin concentration (mg/mL) = (A x BM x FP x 1000)/(E x 1), where A is absorbance, BM is molecular weight (448.8). FP is a diluent factor (5 mL/0.05 mL), and E is an extra molar coefficient (29 600 L/cm). (iii) Measurement of antioxidant activity (Zambiazi et al. 2016). The DPPH radical scavenging activity (antioxidant activity) was determined by using the method described by Zambiazi et al. (2016) with some modifications. The extract solution was prepared by dissolving the extract at concentrations of 25, 50, 100, 200, 400, 800 and 1000 ppm in chloroform: methanol (2: 1). The extracts solution (4 mL) in chloroform: methanol (2: 1) was mixed with 1 mL of DPPH (1,1-diphenyl-2-picrylhydrazyl) solution 0.2 mM in methanol. The mixture was reacted or allowed to stand for 30 minutes before the absorbance showed an increase in DPPH radical capture ability. Uptake was measured by a UV-Vis spectrophotometer at a wavelength of 514 nm. The DPPH radical scavenging activity as a percentage was then calculated as:

DPPH scavenging activity (%) = 100-(A_0-A_1)/A_0 x 100

Where
A_0 = Absorbance from the control or without the addition of sample
A_1 = Absorbance of sample

(iv) Analysis of Vitamin A content (Apriyanto 1989). Chloroform was washed using water in the same or 2-3 times volume, and then the water was released by using anhydrous potassium carbonate. Distillation was carried out, and a small amount (about 10%) of initial distillate was removed. The antimony trichloride solution formed was washed with pure chloroform (water free) until it looked clear. The blank solution was prepared by evenly mixing 4 mL of antimony trichloride reagent and one mL of chloroform. 0.5 mL of the sample solution was then mix with 2 mL of antimony trichloride reagent. Absorbance of the solution was determined by using a spectrophotometer at a wavelength of 620 nm. Vitamin A content was determined based on the standard curve (concentration of vitamin A acetate 0-15 mg/mL).

RESULTS AND DISCUSSION

The levels of Anthocyanin, β-carotene, free radical scavenging activity of sweet potato flour-based biscuits

There was a substantial difference in the effect of antioxidant content and free radical scavenging capacity of the various treatments of biscuits produced from purple, yellow, and white sweet potatoes. The free radical scavenging activity of the treatments of sweet potato flour-based biscuits varied considerably as compared to that of sweet potato flour alone (Table 2).

Roasting of sweet potato flour-based biscuits at a temperature of 150-160° C for 20-30 minutes tended to cause a decrease in the level of their anthocyanin contents as compared to that of the purple sweet potato flour alone. Table 2 shows a tendency of reduction of anthocyanin due to high-temperature roasting during the biscuits production processes. Anthocyanin level in biscuit products decreased from 229.03 ppm (sweet potato flour alone) to, respectively, 95.05 ppm in treatment A1 (58.50%) and 85.20 ppm in treatment A2 (62.80%). The decrease in anthocyanin levels was, presumably, caused by roasting in an oven or heating at a high temperature of 150° C for 20-30 minutes as extended exposure in high temperature can cause degradation of anthocyanin. This is supported by Abidin et al. (2016) who found that steaming with hot steam from boiling water for one hour in the making of steamed *bolu* under treatment of 90% purple sweet potato flour from Kupang District + 10% wheat flour formula produced an anthocyanin level of 19.11 ppm. Additionally, Sari et al. (2005) found that heating at 100° C for 4 hours could reduce the anthocyanin level by up to 80%. Amr and Al-Tamimi (2007) found that 42% and 48% of *Ranunculus asiaticus* anthocyanins were lost after 7 hours of storage at 50 °C and 80 °C, respectively.

Antioxidant activity in purple sweet potato biscuit products decreased from 87.65% (purple sweet potato flour alone) to, respectively, 60.59% in treatment A1 (27.06%) and 61.67% in treatment A2 (25.98%). The decrease in antioxidant activity was, presumably, caused by the decline in anthocyanin levels. Tensiska et al. (2003) found that heating up to 175°C in an aqueous system for 2 hours reduced the activity of up to 17.4%.

Additionally, in their study on osmo-dehydrated blueberries, Giovanelli et al. (2013) reported 50% losses in antioxidant activity after processing. The loss was higher than that reported in the determined total anthocyanin and flavonoid contents. Contrastingly, the current study results showed that the most significant losses occurred in the phenolic compounds, flavonoids, and anthocyanins. According to Xiu-Li et al. (2015), the presence of hydroxyl groups in the anthocyanins molecule may contribute to the color instability caused by heating. Besides, the ring-opening and degradation of anthocyanins were found to be the main factors responsible for the color change at high temperatures. Many studies indicated that the color stability of anthocyanins decreased during storage as temperature rises. The content of β-carotene of biscuit products increased significantly except for biscuits produced from yellow-fleshed sweet potato flour with a tendency to decrease in their β-carotene levels. The antioxidant activity of biscuits also tended to, although not significant (Figures 3 and 4).

Table 2. Levels of anthocyanin, β-carotene and free radical scavenging activity of biscuits produced from purple, yellow and white-fleshed sweet potato from Sumba Barat Daya District

Treatment	Biscuit formulation*	Anthocyanin (ppm)	β-carotene (µg/100 g)	Free radical scavenging activity (%)
A1	50 % pspf+ 50 % wheat flour	85.20	1,745.06	60.59
A2	75 % pspf + 25% wheat flour	95.05	1,881.61	61.57
B1	50 % yspf + 50 % wheat flour	-	9,670.82	52.55
B2	75 % yspf + 25 % wheat flour	-	10,190.44	56.69
C1	50 % wspf + 50 % wheat flour	-	1,197.59	46.47
C2	75 % wspf + 25 % wheat flour	-	1,301.18	46.47
pspf	-	229.03	31.00	87.65
yspf	-	-	26,670.37	86.86
wspf	-	-	16.77	84.51

Note: pspf: purple sweet potato flour, yspf: yellow sweet potato flour, and wspf: white sweet potato flour; -: unreadable tool

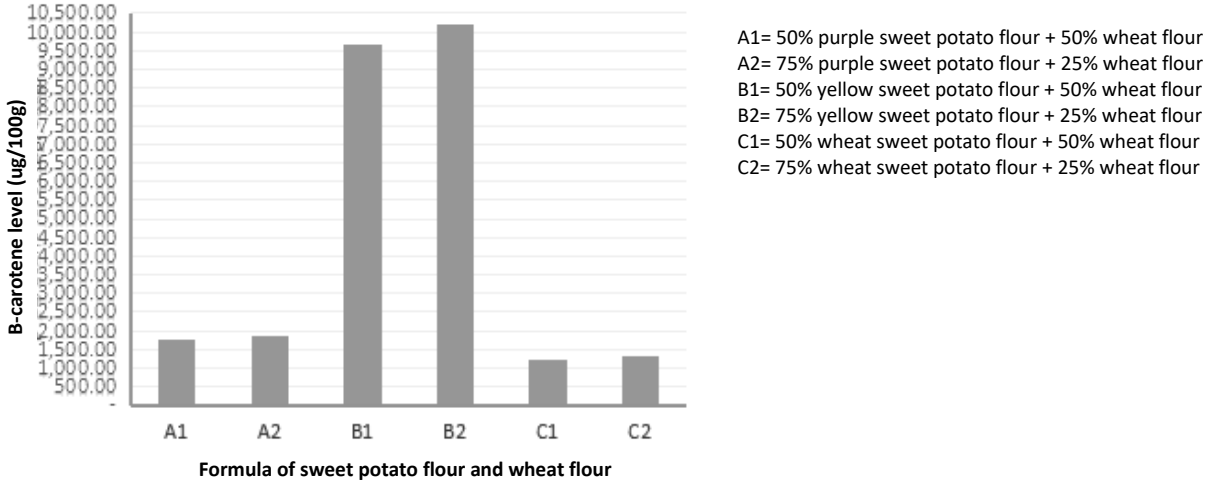

Figure 3. The effect of roasting on β-carotene content of biscuits produced from purple, yellow and white sweet potatoes flour

Figure 4. The effect of roasting on free radical (DPPH) scavenging capacity (antioxidant activity) of biscuits produced from purple, yellow and white sweet potatoes flour

It is demonstrated in Figure 3 that β-carotene levels significantly increased in biscuits produced from purple and white sweet potato flour as compared to that of plain purple and white sweet potato flour alone (31 µg/100 g and 16.17 µg/100 g). The increase of β-carotene levels was caused not only by additional β-carotene from sweet potato flour but also by the use of blue band butter in biscuit formulas. Butter contains natural antioxidants in the form of beta carotene of 30% (PT Unilever Indonesia 2018). β-carotene levels in biscuits produced from yellow sweet potato tended to decrease from 26670.37 µg/100 g (yellow sweet potato flour alone) to, respectively, 9670.82 µg/100 g in treatment B1 and 10190.44 µg/100 g in treatment B2. Biscuit products produced from yellow sweet potato flour experienced a decrease in β-carotene levels as the carotene was degraded by heating during roasting in the oven. Beta carotene level in yellow sweet potato flour-based biscuits was 40-47 times higher than that of purple and white sweet potato flour biscuits. Thus, yellow sweet potato flour can be used as one of the functional food ingredients. Yellow sweet potato contains a high β-carotene level. About 89% of total carotene in yellow sweet potato is in the form of β-carotene, 96.7% of which is dominated by the trans-β-carotene form (Sweeney and Marsh 1970; Woolfe 1992).

Additionally, Kurniawati and Ayustiningwarno (2012) stated that β-carotene content can be reduced and damaged due to processing. The existence of a double bond structure in β-carotene causes β-carotene to be easily oxidized when exposed to air (O_2). Erawati (2006) found that the oxidation process will take place more quickly in the presence of light, metal catalysts, and heating processes at high temperatures. This can result in a change in the trans-β-carotene structure to cis-β-carotene, where it has a lower provitamin A activity. Also, Lisia (2001) found that the

content of β-carotene in yellow sweet potato flour was also reduced because of processing in bread making. Furthermore, Yusianti and Hariyadi (2001) also found that yellow sweet potato flour bread showed a decrease of β-carotene by 68.5% during roasting in an oven at ± 160° C for 15 minutes. The level of the reduction will be higher with increasing temperature and roasting time.

Vitamin A is an essential nutrient that cannot be produced by the human body alone and is needed in limited quantities. Vitamin A possesses health benefits, i.e., in the process of vision, growth, reproduction and protecting cells and tissues from the effects of damaging free radicals leading to degenerative diseases (Mukherjee and Mitra 2009). Vitamin A deficiency may lead to fetal development abnormalities, anemia, weak immune function, rusting of the respiratory tract, and digestive tract, urinary tract, skin, and eye epithelium disorders (Mahan and Stump 2004). The effect of vitamin A deficiency is not detrimental at the beginning, but the untreated patient may suffer dusk blindness (*xerophthalmia*), where the sufferer is unable to see in the gloomy light. If this disease continues, it will cause the cornea to turn yellow, and a pattern appears on the cornea, which may cause the patient to suffer permanent blindness. Vitamin A deficiency in children may cause measles, respiratory infections, and diarrhea. The primary cause of vitamin A deficiency is a low intake of vitamin A-containing nutrients or vitamin A precursors that may not suffice the needs of vitamin A in certain physiological and pathological conditions. Frequent suffering of diarrhea may also lead to low absorption of vitamin A.

It is depicted in Figure 4 that antioxidant activity or DPPH free radical scavenging activity of sweet potato-based biscuit products was high, ranging from 46.47-61.57%. The free radicals scavenging activity in both products was generally higher than that of black rice from Ende, which ranged from 46.06-53.43% (Lalel et al. 2009). The free radical scavenging activity is the most essential factor possessed by functional food. Free radicals originated from various sources such as pollutants or waste from the surrounding environment, food processing byproducts and motor vehicle and industrial pollution. These free radicals are very reactive to attack the molecules contained in the cell system, causing damage that cannot be repaired by the cells. Reactive oxygen species (ROS) damages lipids, proteins, enzymes, carbohydrates, and DNA in cell membranes leading to aging, heart disease, and cancer (Pietta 2000).

Sweet potato flour-based biscuits products are also considered functional foods as they contain functional factors. Antioxidant compounds in the form of anthocyanin and vitamin A contained in both products possess characters of chelating and preventing the effect of free radicals in the human body. Oxidation reactions are essentially chemical reactions that occur in the human body. The problem will arise from oxidation if the electron flow becomes unpaired resulting in free radicals including ROS such as superoxide (O_2*), peroxide (ROO*), alkoxyl (RO*), hydroxyl (HO*) and nitric oxide (NO*). The very short half-life of free radicals such as hydroxyl (10-9 seconds) and alkoxyl (a few seconds) cause them to very reactive and rapidly attack molecules in nearby cells leading to unrepaired damage in the cell system. ROS can even be very damaging as it can attack lipids in cell membranes, tissue proteins or enzymes, carbohydrates, and DNA, causing damage to cell membranes, enzymes, and DNA. This oxidative reaction has been considered to play a role in the aging process, and various degenerative diseases such as heart attacks, cataracts, cognitive abilities dysfunction, and cancer (Pietta 2000).

β-carotene is an antioxidant, a provitamin A that is converted into vitamin A in the human body. It is highly beneficial for growth, maintenance of body tissues and vision, reproduction, fetal development, and reducing the risk of cancer and liver disease (Keller 2001).

To conclude, purple, yellow, and white sweet potatoes flour-based biscuits can be consumed as functional food. Biscuits produced from purple sweet potato contained 95.05 ppm anthocyanin, 1881.61 µg/100g β-carotene and high free radical scavenging activity (61.57%). Yellow sweet potato flour-based biscuits contained the highest β-carotene content (10190.44 µg/100 g) and free radicals scavenging ability of 56.69%.

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Various dosages of active powder of cassava improved sustainability of physical and chemical characteristics of Vertisol and Alfisol on dryland farming system

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Abstract. *Soetedjo IP. 2019. Various dosages of active powder of cassava improved sustainability of physical and chemical characteristics of Vertisol and Alfisol on dryland farming system. Trop Drylands 3: 29-33.* Vertisol and Alfisol are two types of dominant soil in dryland farming systems. Both soils generally have a low content of some nutrients such as N, P, K, C organic, and are dominated by clay, high water saturation, and prone to Al, Fe, and Mn poisonings. An innovative product called active powder may improve soil microbiology activity and physical characteristics of soil, which might then improve the availability of soil nutrients. Research had been done to know the effect of various dosages of active powder to enhance the physical and chemical characteristics of Vertisol and Alfisol in dryland farming systems in Kupang, East Nusa Tenggara, Indonesia. The research was a factorial treatment design laid out in a Randomized Complete Block Design with four replicates for each treatment. The treatments employed were two types of soil (Vertisol and Alfisol) and various dosages of active powder (0, 100, 200, and 300 g ha^{-1}). Parameters observed were subjected to ANOVA and was followed by a Least Significant Different Test at 0.05 level. Results of the research showed that dosage of active powder of 300 g ha^{-1} was able to improve the number colony of soil bacteria, soil porosity, soil bulk density, total N, P availability, and K availability. Generally, the physical and chemical characteristic of Vertisol was better improved than Alfisol. The yield of mungbean was significantly affected by the dosage of the active powder of 200-300 g ha^{-1} in which yield of mungbean grown at Vertisol was higher than that at Alfisol.

Keywords: Alfisol, dosage, active powder of cassava, physical and chemical characteristic of soil, vertisol

INTRODUCTION

Alfisol and Vertisol are two types of soil that dominantly occurred in dryland areas such as in East Nusa Tenggara, Indonesia. Alfisol has low content of nutrients such as N, P, K, C organic, Mo, Mg, and poisoning levels of Al, Fe, and Mn. Moreover, Alfisol has medium to high bulk density, less to medium soil permeability, low water soil resistance, high sensitivity to erosion, and low content of soil microbiology. Similarly, Vertisol has a low content of some nutrients such as N, P, K, C organic, dominated by clay, and high water saturation (Hardjowigeno 2015; Serangmo 2015). Those characteristics result in low soil quality, limiting the capabilities of the soil in supporting growth and yield of crops. Numerous ways have been practiced to improve the capabilities of Vertisol and Alfisol, such as applying organic and organic fertilizers. However, several studies showed that those practices resulted in the increase of soil compaction, and increasing of soil microbiology dormancy (Debosz, et al. 2001; Abebe 2001; Esmaeilzadeh and Ahangar 2014).

Soil microbiology elements are needed to improve physical soil characteristics and improve some soil nutrient availability. Previous studies showed that organic fertilizer could improve the availability of soil microorganisms and the availability of nitrogen, phosphorus, and potassium (Doran and Zeiss 2000; Debosz et al. 2001; Abu-Zahra and Tahboub 2008). However, it needs a great quantity of organic fertilizer, about 10 - 20 t ha^{-1}. This quantity is hardly fulfilled in dryland farming system practices, which is generally limited in sources of organic matter and manpower. Therefore, it is necessary to find innovative technologies that could minimize the use of organic sources, but could improve the physical and chemical characteristics of soil.

The active powder is one of innovative products that are available to improve soil microbiology activities, which then improve the availability of some soil nutrients. Physically, the active powder may be able to maximally increase the use of gamma radiation to increase the number of oxygen in the soil. Gamma radiation can intercept into 50 cm soil depth, which increases the degradation of H_2O becomes hydrogen (H_2) and oxygen (O_2). Increased soil oxygen levels will increase the activity and biomass of soil microorganisms, which may stimulate the improvement of soil structure, soil aeration, exchange capacity of anions and cations, and nutrient availability. These positive effects, however, will strongly depend on soil type, the dosage and time of application, and plant growth, which are mainly due to various types of soil that have different physical and chemical characteristics as reported by Esmaeilzadeh and Ahangar (2014) and Hardjowigeno (2015). This research aimed to know the effect of various

dosages of active powder of cassava on enhancing the physical and chemical characteristics of Vertisol and Alfisol in dryland farming systems in Kupang, East Nusa Tenggara, Indonesia.

MATERIALS AND METHODS

Research location and materials

The present research was conducted at Naibonat Village (dominated by Vertisol) and Matani Village (dominated by Alfisol), Kupang District, East Nusa Tenggara Province, Indonesia from May to October 2017. Materials used in the research were active powder of cassava and mungbean plants.

Research design

A factorial experimental design was employed, laid out in a Randomized Complete Block Design with four replications. The assigned treatments consisted of two soil types (Vertisol and Alfisol) as the first factor and dosages of active powder (0, 100, 200 300 g ha^{-1}) as the second factor. The active powder was applied during planting.

Experimental procedures

Active powder of cassava was prepared two days before application into the soil. All active powder of cassava was mixed with 2 kg ha^{-1} of wheat flour and thereafter mixed with 4 liters of water. Mungbean was grown on planting plot of 2 m x 6 m with planting space of 20 cm x 40 cm.

Three soil samples were collected before planting at 0-20 cm soil depth from each replicate area by using an auger and were mixed to form a composite sample for each treatment. Each soil sample was mixed, then four replications were taken for measurements and analysis and the average readings were recorded. Besides, at the end of the experiment soil samples were collected in the same manner, as before planting, but replicates of each treatment were not mixed with each other, and analysis was done on the four replicate samples for each treatment, then average readings were recorded.

Observation and data analysis

Observed parameters included the number of the colony of soil bacteria, nutrients content (Nitrogen, Phosphorus, Potassium, C organic), and yield of mungbean. Soil chemical characteristic and number of colony soil bacteria of sample soil were analyzed before planting and after harvest. Total nitrogen (N) was determined by using a micro Kjeldahl method (Bremner and Mulvaney 1982). Available phosphorus (P) was extracted by 0.5 N NaHCO with pH of 8.5 with Spectrophotometer (Olsen and Sommers 1982). Available potassium (K) was extracted by 1 N ammonium acetate determined by Flame Photometer (Knudsen Peterson and Pratt 1982).

The yield of mungbean was observed at the end of the research by weighting dry seeds harvested per plant and per plot. The treatment means were separated by using the Least Significant Difference at 5% significant level.

RESULTS AND DISCUSSION

Soil bacteria colony

Result of this research showed that increasing the application dosage of active powder up to 300 g ha^{-1} resulted in an increase in the number of soil bacteria colonies (Table 1) on both soil types (Vertisol and Alfisol). This is mainly because those soils have a clay content, low soil porosity and high bulk density. These conditions resulted in low content of Oxygen that soil microorganism activities highly require it. By the application of active powder up to 300 g ha^{-1}, the available oxygen becomes higher, which may able to increase the number and activities of soil microorganisms. This will then result in improved soil porosity, improved soil aeration, decreases in bulk density, and improvement of cation exchangeable capacity. Several studies reported that the availability of soil microorganisms depends strongly on soil microclimatic conditions such as pH, aeration, energy sources and soil management (Hassink 1994; Papadopoulos et al. 2006; Jangid 2008).

Moreover, the research showed that number of bacteria colonies was higher at Vertisol than Alfisol (Table 2). This is mainly due to the percentage of porosities on Vertisol was relatively higher than Alfisol at the various dosages of active powder applications. This significantly affected an increase of oxygen level and increase the source of energy required by soil microorganism activities. Hassink (1994); Papadoulos (2006), and Soetedjo (2018) reported that availability of nutrients, well aeration and drainage, better source of energy (solar radiation and organic matter) are several factors that should be taken into account to improve soil microorganism growth and development.

Table 1. The effect of various dosages of active powder application on the number of soil bacteria

Active powder dosage (g ha^{-1})	Number of soil bacteria colony			Changeable number of soil bacteria colony	
	Before active powder application	After active powder application			
0	236.5	233.50	a	3	a
100	236.5	271.37	b	34.87	b
200	236.5	285.25	bc	48.75	bc
300	236.5	292.87	c	53.37	c
LSD 5%		15.23		15.23	

Note: Numbers followed by the same letter at the same column means not significantly different at LSD 5% level

Table 2. Number of soil bacteria at different soil types as affected by various dosages of application

Soil type	Number of soil bacteria colony		Changeable of Number of soil bacteria colony
	Before application	After application	
Vertisol	261.5	277.63 b	25.68 a
Alfisol	211.5	263.88 a	52.38 b
LSD 5%		10.77	10.77

Note: Numbers followed by the same letter at the same column means not significantly different at LSD 5% level

Soil porosity

Result of the present research showed that active powder application significantly affected soil porosity. Applications of 100 - 300 g ha^{-1} active powder resulted in increase soil porosity of Vertisol and Alfisol as compared to no application of active powder (Table 3). These results showed that active powder can improve oxygen level on the soil, which resulted in increasing soil microorganism activities to improve soil porosities, soil aeration, and decrease bulk density (Papadopoulus et al. 2006; Jangid et al. 2008; Soetedjo 2018). Moreover, improvement of soil porosities of Vertisol was higher than soil porosities of Alfisol. Some studies by Granatstein et al. (1987), Hassink (1994), and Soetedjo (2008) reported that activities and biomass of soil microorganisms significantly increased soil physical properties of low clay content than high clay content (Table 4).

Total nitrogen content

There was no interaction effect of the application dosage of active powder and type of soil on Nitrogen content. However, the single factor dosage and type of soil significantly affected Nitrogen content. LSD test at level 5% showed that active powder application up to 300 g ha^{-1} resulted in an increase in Nitrogen content of both types of soil, where the rise of Nitrogen content on Vertisol was significantly higher than that on Alfisol (Table 5).

An increase in nitrogen content at 300 g ha^{-1} of active powder application is mainly due to the increased number of microorganisms that resulted in improvement of soil micro-bacteria activities, improvement of soil porosity, decreasing in soil colloid and improvement of exchangeable cation capacity. Several studies by Granatstein et al. (1987), Wild. (1993), and Beja et al. (2015) reported that increase in number and activities of soil microorganisms as result of various soil management improved the availability of some nutrients (N, P, and K). Improvement of Vertisol is much better than Alfisol that this could be related to the physical characteristic of Vertisol, which is better in supporting gamma radiation interception into the deeper part of the soil resulted in increased number of soil microorganisms on the soil (Serangmo 2015).

Available phosphorus

The ANOVA result revealed a significant interaction effect of active powder dosage and type of soil on available phosphorus. LSD post hoc test showed that all dosages of the active powder application on Alfisol resulted in higher content of available phosphorus as compared to that of Vertisol (Table 6). This is mainly because a higher Nitrogen content on Vertisol suppressed the amount of phosphorus in the Vertisol. Meanwhile, both soil types had a high content of available Phosphorus at 300 g ha^{-1}of active powder application. These results showed that there is significant correlation between improvement of the availability of some nutrients with increased number of soil microorganisms, and improved of porosities soil aeration (Hassink 1994; Papadoulos 2006).

Table 3. The effect of various dosages of active powder application on soil porosity

Active powder Dosage (g ha^{-1})	Soil porosity affected by active powder application		Changeable of soil porosity
	Before	After application	
0	59.65	58.81 a	-0.84 a
100	59.65	62.97 b	3.32 b
200	59.65	63.06 b	3.41 b
300	59.65	64.97 b	5.32 b
LSD 5%		3.39	3.39

Note: Numbers followed by the same letter at the same column means not significantly different at LSD 5% level

Table 4. Soil porosity levels at different soil types affected by various dosage applications

Soil type	Soil porosity affected by active powder application		Changeable of soil porosity
	Before	After application	
Vertisol	55.01	60.73 a	5.74 b
Alfisol	64.3	64.15 b	-0.14 a
LSD 5%		2.40	2.40

Note: Numbers followed by the same letter at the same column means not significantly different at LSD 5% level

Table 5. The effect of the single factor active powder application and type of soil ton total nitrogen content (%)

Active powder dosage (g ha^{-1})	Before application	After application	Changeable of nitrogen content
0	0.067	0.57 a	0.51 a
100	0.067	0.65 bc	0.58 ab
200	0.067	0.66 c	0.59 b
300	0.067	0.71 c	0.64 b
LSD 5%		0.07	0.08
Vertisol	0.067	0.81 b	0.74 b
Alfisol	0.067	0.49 a	0.42 a
LSD 5%		0.05	0.05

Note: Numbers followed by the same letter at the same column at the different single factor of active powder dosage and soil type means not significantly different at LSD 5% level.

Table 6. The effect of interaction between the dosage of active powder application and type of soil on available phosphorus content (ppm)

Type of soil/ active powder dosage (g ha^{-1})	300	200	100	0
Vertisol	16.0975 b A	15.88 ab A	15.4225 ab A	14.81 a A
Alfisol	22.7175 b B	21.64 ab B	20.3875 ab B	19.427 a B

Note: Numbers followed by the same small letter at the same row and capital letter at the same column means not significantly different at 5% LSD level.

Potassium content

Result of the research showed no significant interaction effect of dosage of active powder application and type of soil on Potassium content. However, the single factor treatments affected the Potassium content of the soil. The application of 200-300 g ha^{-1} of active powder significantly increased the Potassium content of both soil types; meanwhile, the Potassium content of Vertisol was higher than Alfisol (Table 7). All the results showed that improvement of the physical characteristic of the soil directly affected the nutrient content of the soil. A better increase in soil porosity resulted in increased number of soil microorganism and their activities, which stimulate increase in cation expandable capacity and availability of nutrients (Doran and Zeiss 2000; Habig and Swanepoel 2015.

C organic content

Result of the research showed no significant interaction effect of active powder and soil types on C organic content. However, the single factor soil type significantly affected C organic content. C organic content of Alfisol was higher than Vertisol that is mainly because Vertisol has a higher clay textured soil than Alfisol. In addition, increase in Nitrogen content affected by powder active application could cause a decrease in C organic content (Table 8). Several studies showed that physical characteristics of soil (texture, structure, aeration, bulk density) and chemical characteristics of soil (Total N content) were closely related to C organic content. High clay-textured soil such as Vertisol has a low C organic content, and a high content of N on Vertisol will suppress the organic content as reported by Hessink (1994);) and Abu-Zahra and Tahboub (2008).

Table 7. The effect of single factors active powder application and type of soil on potassium content (me/100g)

Active powder dosage (g ha^{-1})	Before application	After application	Exchangeable of potassium content
0	0.66	0.69 a	0.03 a
100	0.66	0.99 b	0.34 a
200	0.66	1.07 bc	0.41 b
300	0.66	1.16 c	0.50 b
LSD 5%		0.11	0.11
Vertisol	1.21	1.20 b	-0.02a
Alfisol	0.11	0.75 a	0.65 b
LSD 5%		0.08	0.05

Note: Numbers followed by the same letter at the same column at different single factor treatment of dosage and type of soil means not significantly different at 5% LSD.

Table 8. The effect single factor type of soil on C organic content of the soil (%) affected by active powder application

Type of soil	Before application	After application	Exchangeable
Vertisol	1.26	0.44 a	-8.11 a
Alfisol	1.05	1.15 b	0.11 b
LSD 5%		0.53	0.56

Note: Numbers followed by the same letter at the same column means not significantly different at LSD 5% level

Table 9. The effect of single factor active powder application and type of soil on the yield of mungbean (g/ 6 m^2)

Active powder dosage (g ha^{-1})	Before application	After application of active powder
0	0	166.38 a
100	0	170.50 ab
200	0	201.88 b
300	0	225.38 b
LSD 5%		35.19
Vertisol	0	247.44 b
Alfisol	0	134.63 a
LSD 5%		24.88

Note: Numbers followed by the same letter at the same column at the different single variable of dosage and type of soil means not significantly different at LSD 5%l.

Yield of mungbean

Result of the research showed that the yield of mungbean was significantly affected by the single factor treatments of dosage application of active powder and type of soil. The application of 200-300 g ha^{-1} active powder resulted in a higher yield of mungbean (g/6 m^2) as compared to that of no application of active powder. Moreover, the yield of mungbean on Vertisol was higher than that of Alfisol. Higher mungbean yield on Vertisol was mainly due to the improvement of physical and chemical characteristics of the Vertisol was better than Alfisol (Table 9).

In conclusion, the application of active powder significantly improved soil physical and chemical characteristics of Vertisol and Alfisol. The application of 300 g ha^{-1} active powder resulted in a better physical and chemicals characteristic of Vertisol as compared to Alfisol. A higher yield of mungbean (225 g per 6 m^2) was obtained by application of 300 g ha^{-1} active powder.

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