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Evaluation of regeneration potentials of farmer-preferred cassava (Manihot esculenta crantz) landraces to unlock cassava transformation barriers

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ABSTRACT: Evaluation of cassava germplasms for in vitro regeneration ability is crucial for stable genetic improvement of this crop via genetic transformation systems. Methods for reliable and efficient transformation including somatic embryo regeneration and recovery of transgenic plants still need to be developed and customized for each cassava genotype. Twenty one Tanzanian farmer-preferred cassava (Manihot esculenta Crantz) landraces widely grown in major cassava growing zones were evaluated for somatic embryo induction, recovery, sustainability and plantlets regeneration to whole plants. Somatic embryogenesis was induced from cassava leaf lobes on Murashige and Skoog media supplemented with different concentrations of sucrose, Copper sulphate and 2, 4-Dichlorophenoxyacetic acid and further developed to plantlets. Frequency of somatic embryo production and subsequently regeneration stages were evaluated starting from 28 days post-inoculation. All cassava genotypes tested in this study were able to induce callus where by 62 % were able to induce somatic embryo cotyledons. Cassava landraces: Sagalato, Rangimbili, Mnazi and Kibandameno were highly responsive to somatic embryo production. The conversion rate of embryos into plantlets was variable depending on the cassava landrace, Sagalato being the most responsive and Kiroba the least. Somatic embryos from 8 cassava landraces reached plantlet stage, 5 of them being acclimatized and successfully developed to plants with normal phenotype and they rooted on soil. The regeneration potentials of farmer-preferred cassava landraces observed in this study is hoped to pave a way towards genetic improvement for both biotic and abiotic stresses via genetic engineering approaches.
Key words: Friable calli; 2,4-D; MARI; Somatic embryo; Tanzania
Abbreviations: BAP-6, benzylaminopurine, 2, 4-D- Dichlorophenoxyacetic acid, FECs-Friable embryogenic calli, MARI-Mikocheni Agricultural Research Institute, MS- Murashige and Skoog, NAA-Naphthalene acetic acid

INTRODUCTION

Cassava (Manihot esculenta) is a tuberous root crop that serves as a source of carbohydrate for more than 800 million people worldwide and its cultivation is estimated to cover more than 18.9 million hectors (FAO, 2011). It is a vital source of food and income to resource poor farmers (especially women) in the tropics and staple food crop for nearly 200 million people in the sub-Saharan Africa (Liu et al., 2011; Nyaboga et al., 2013). In Tanzania, cassava ranks only second to maize and third to rice, with its calories contribution per day being 15 % (FAO 2009).

Cassava production in Africa is constrained with various pests and viral diseases especially cassava brown streak and cassava mosaic diseases. Research efforts to genetically improve resistance against viral diseases, drought, postharvest deterioration and nutrients fortification of this crop are underway (Zang et al., 2005; Vanderschuren et al., 2009; Nyaboga et al., 2013). All these efforts are through genetic transformation method which relies on the ability of cassava to regenerate from somatic embryos to full plant. Due to heterozygozity nature of cassava, it is difficult to use its zygotic embryos as starting materials for genetic transformation protocols (Raemakers et al., 1997; Sarria et al., 2000; Taylor et al., 2001; Siritunga and Sayre 2003). Instead, organised structures from somatic embryo cotyledons and friable embryogenic calli (FECs)

have been developed (Schöpke et al., 1996; Taylor et al., 2004). FECs are the best organized structures to use as starting materials for genetic transformation (Vanderschuren et al., 2009; 2012) but in many laboratories production of FECs from farmer- preferred cultivars is still a big challenge (Taylor et al., 2001; 2004; Hankoua et al., 2006; Nyaboga et al., 2013). Protocols for production of FECs is very expensive in terms of labour, space and high skills (Taylor et al., 2004; Hankoua et al., 2006; Vanderschuren et al., 2009) thus a challenge in various laboratories in developing world.

In contrary, a protocol for production of somatic embryo cotyledons is relatively short hence less labour intensive. It requires neither large space nor frequent change of media thus inexpensive as compared with FECs. Although somatic embryo cotyledons are less effective when used as starting materials for transformation, yet its potential cannot be neglected. With limited resources and relatively little skilled staff, somatic embryo cotyledons can be produced and used for cassava transformation especially in less developed countries. Furthermore, FECs protocol passes through somatic embryogenesis stage and when subjected to Doy and Greshhoff media, the indirect somatic embryogenesis-friable embryogenic callus occurs (Taylor et al., 2001). Therefore, evaluating and establishing regeneration potential of farmer-preferred cassava landraces via somatic embryogenesis is important before attempting genetic transformation of this crop. However, somatic embryogenesis is highly genotype dependent and therefore, screening important Tanzania cassava genotypes for their embryo induction and regeneration ability represents an important aspect towards a successful genetic transformation. In addition, focus of most cassava tissue culture regeneration efforts have largely been on cassava varieties from South America, model variety cv. 60444 and a few West African cultivars. Challenges still remain on moving and customizing the transformation protocol to accommodate various potential African farmer-preferred cassava cultivars.

This study evaluated cassava genotypes preferred by farmers due to their good agronomic traits such as high yield, early maturity, tolerance to diseases and good ground storability among others, on their ability to induce somatic embryo cotyledons, regeneration and frequency of plant recovery. Amenability of farmer-preferred cassava landraces to regeneration protocols will pave a way for alternative improvement of this crop to manage both biotic and abiotic stresses via genetic modification approaches.

MATERIALS AND METHODS

PLANT MATERIAL

All cassava landraces used in this study (were also used in another study by Elibariki et al. 2013) were obtained from cassava farmers in Tanzanian major cassava agro ecological zones (Table 1and Figure 1). During sampling, farmers provided a common name of each cassava landrace, an origin (if known) and its agronomic traits. About 25-30 cm stakes with at least two viable nodes were obtained from healthy mature plants and were planted in seedling pots in a screen house at Mikocheni Agricultural Research Institute (MARI), Tanzania. The stakes were then watered for about two weeks for sprouting and the sprouts were used for micro propagation on MS salts (Murashige and Skoog 1962).

Micropropagation

Sprouts with at least 4 nodes were harvested from the tip for initiation of in vitro explants. Shoots obtained were washed with sterile distilled water, soaked in 70 % ethanol for 5 minutes then rinsed with sterile distilled water. Two drops of hypochlorite were added for 20 seconds and rinsed with sterile distilled water. All cassava landraces were multiplied in vitro through nodal segments on standard cassava micropropagation medium comprising of MS salts with B5 vitamins. The media was supplemented with 2 % (w/v) sucrose, 0.02 mg/L α-naphthalene acetic acid (NAA), 0.04 mg/L 6-benzylaminopurine (BAP) and 0.05 mg/L giberellic acid (GA_3). Shoots were maintained on a 12 hours photoperiod at 28 °C.

Induction of somatic embryogenesis of all cassava landraces

Two weeks old explants of all each cassava landraces were removed from the media and examined for young unopened leaves (immature leaf lobes). The young unopened leaves were cut using a pair of scissors and chopped at the bottom and at the apex on a sterile filter paper. The middle potion of the leaf was placed on a standard MS media supplemented with 2 % sucrose, B5 vitamins (100 X stock), 1.5 mM $CuSO_4$, Casein hydrolysate and 16 mg/l of 2,4-D. Each plate was inoculated with 5-10 leaves. The plates were double-sealed with parafilim and some were covered with aluminium foil (only for dark treatment) and were incubated at 28ºC under 16 hours photoperiod for about 4-8 weeks to produce somatic embryo cotyledons. Somatic embryos produced from each cassava were counted and recorded at 3 stages: globular, cotyledonary and mature stages. In all experiments described in this study, frequency of somatic embryo induction was recorded using a scale of 0-5 as described by (Taylor et al., 1996) whereby 5 represents somatic embryos covering the entire margin of the explant and zero means no somatic embryo. Three somatic embryogenic responsive landraces

were chosen for further experiments under optimized media by varying concentrations of 2, 4-D, sucrose and copper sulphate.

Somatic embryo induction on selected landraces under optimized 2, 4-D concentrations

To determine the response of cassava genotypes under various 2, 4-D concentrations on the somatic embryogenesis, three landraces that were among the most responsive to somatic embryo induction: Sagalato, Kibandameno and Rangimbili were used. Explants of the three cassava landraces were used and cultured as described in the previous section using MS media but the 2, 4-D concentrations were varied to 4, 6, 8, 12, or 14 mg/l. Each plate was inoculated with 8 leaves (explants) and the treatment replicated three times. The plates were double-sealed with parafilim and incubated at 28 °C under 16 hours photoperiod for about 4-8 weeks to produce somatic embryo cotyledons.

Somatic embryo induction on selected landraces under optimized sucrose concentrations

Two weeks old explants of three cassava landraces (Sagalato, Kibandameno and Rangimbili) were inoculated on a standard MS media except that the sucrose concentration was varied to either 4 % or 6 %. Each plate was inoculated with 8 explants and the treatment replicated three times. All plates were double-sealed with parafilim and incubated at 28 °C under 16 hours photoperiod for 4-8 weeks.

Somatic embryo induction under optimized copper sulphate concentrations

A standard MS media supplemented with 0 mM, 1.0 mM, 1.5 mM or 2 mM of copper sulphate were used. Two weeks old explants of 8 somatic embryogenic responsive cassava landraces were used. Each plate was inoculated with 8 explants and the treatment duplicated. All plates were incubated at 28 °C under 16 hours photoperiod for about 4-8 weeks to produce somatic embryo cotyledons.

Response of representative cassava landraces on induction of somatic embryos under limited light

Seven cassava landraces were randomly selected and their explants inoculated on MS media supplemented with B5 vitamins (100 X stock), Casein hydrolysate, 16 mg/l of 2, 4-D and 2 mM copper sulphate. Each plate was inoculated with 10 explants, double-sealed with parafilim and covered with two layers of aluminium foil and incubated at 28 °C under 16 hours photoperiod for about 4-8 weeks.

Germination and plant regeneration

Mature somatic embryos (from all treatments) showing distinct root and shoot axes were transferred into baby jar tissue culture flasks containing MS medium with vitamins supplemented with 2 % (w/v) sucrose (pH 5.7-5.8) and 0.2 % (w/v) Phytagel for solidification. The cultures were maintained at 28 °C, exposed to a daily photoperiod of 12 hours with general electric cool white fluorescent tubes until they have developed enough leaves and roots. Mature plants were sub cultured on the same media using nodal segments and maintained under the same condition for 4 weeks or until they were mature enough to be transferred into soil. For acclimatization, small seedling pots (diameter=10 cm) were filled with coconut fibres (substrate) sprinkled with distilled water and 0.5 g/l polyfeed starter (liquid fertilizer). Excess media was removed carefully from plants roots and using sterile forceps the plants were transferred on the substrate for acclimatization. The pots were covered with white polythene bags to maintain humidity and after 7 days a small hole was made on top of the polythene bags to allow little aeration to the plants. The plants were maintained in the screen house for 7 days. A sterile mixture of loam soil, sand and manure was prepared in the ratio of 3:2:1 respectively. About 2 kg of the soil mixture was packed in black polythene bags (diameter 22 cm) and watered. Plants from acclimatization pots were carefully transferred into the soil and maintained in the screen house for eight weeks for scoring the recovered plants.

RESULTS

Somatic embryogenesis response of all cassava landraces

All 21 cassava landraces were able to induce callus. Thirteen out of 21 cassava landraces (62 %) were amenable to somatic embryogenesis. Time required to induce somatic embryos and to attain cotyledonary stage varied among the genotypes. Cassava landraces namely Sagalato, Rangimbili, Kibandameno, Paja la Mzee and Mnazi were among the most responsive to somatic embryo induction compared with the rest.

Response of selected cassava landraces under optimized 2, 4-D media

All three cassava landraces which were the most responsive to somatic embryo induction on the standard media responded well to the optimized 2, 4-D media. However, under very low concentrations of 2, 4-D (4 and 6 mg/L), all three cassava landraces produced relatively small calli with very few embryos compared with their response to the media with 8-14 mg/l of 2, 4-D (data not shown). The primary somatic embryos

emerged on the 4th week post inoculation with Sagalato being most responsive under all treatments followed by Rangimbili as depicted in Figure 2.

Response of selected cassava landraces under optimized sucrose concentration

The best three somatic embryogenic competent cassava landraces performed variably on various levels of sucrose (Figure 3). Cassava landrace Sagalato was the most responsive in all sucrose levels with higher number of embryos observed at 6 % sucrose concentration. However, at 6 % sucrose concentration entire calli of all genotypes turned to brown and the embryos turned white after 4 weeks.

Response of selected cassava landraces under optimized Copper sulphate media

Among 8 tested landraces, only Kibandameno could induce somatic embryo on the media without copper sulphate. The rest cassava landraces could not induce somatic embryos in the media completely lacking Copper sulphate. However, each landrace responded differently on various concentrations of Copper sulphate Rangimbili being the most responsive at 1.5 mM concentration followed by Sagalato and Mnazi while Kiroba and Kaniki were the least responsive as indicated in Figure 4.

Response of cassava landraces to somatic embryo induction under limited light

Seven randomly selected cassava landraces that were assessed in this experiment demonstrated that in the absence of light somatic embryo responsive landraces could still induce embryos. On the other hand, the less responsive cassava landraces ended up with callus of varying structure and size but no embryo produced as indicated in Table 2. One cassava landrace named Gago were observed to be more responsive under limited light.

Regeneration and plantlets recovery

Regeneration was achieved from all 21 landraces whereby all were able to induce callus but each landrace responded differently on further developmental stages. Regeneration potential of the tested landraces was determined at three important stages namely globular, cotyledonary and plantlet. The developmental stages achieved by each cassava landraces is summarized in Table 3 and regeneration regime of 2 selected landraces are shown in Figure 5 and 6

DISCUSSION

The most important step before cassava transformation for any important agronomic trait is the production of organised, primary embryogenic tissues from the explant. This is possible through establishment of efficient regeneration protocol in order to eventually produce transgenic plants through various systems. Since genetic transformation of cassava depends on regeneration ability of the cultivar in question, and since each cassava genotype responds differently to each regeneration protocol (Raemarkers et al., 1997; Taylor et al., 2001; 2004; Ihemere, 2003; Saelim et al., 2006; Hankoua et al., 2006; Atehnkeng et al., 2006), it was essential to optimize a protocol for efficient somatic embryogenesis of Tanzanian cassava landraces.

In this study, various factors known to have an effect on the cassava somatic embryogenesis and regeneration were evaluated. The source and concentration of auxin plays a role in the regeneration of various plants. This study determined higher levels of 2, 4-D as best inducers of somatic embryos. The results of this study greed with previous study by Saelim et al., (2006) who evaluated the effect of 2, 4-D, dicamba and picloram on the somatic embryogenesis of two Asian cassava cultivars and found 2, 4-D to be the best inducer at 12 mg/l. Similarly, Ihemere (2003) determined also higher number of cassava somatic embryos produced under 12 mg/l of 2, 4-D. This study observed that 2, 4-D at 12 to 16 mg/l provided better results for those cassava landraces which were responsive to somatic embryo induction. However, Fletcher et al. (2011) observed 8 mg/l of 2, 4-D as the best concentration for callus induction in four Ghanaian cassava cultivars. Other factors like the source and health status of the explants used in this study may have contributed to results obtained.

Response of selected landraces on an optimized sucrose concentration on the somatic embryogenesis was also variable. Although at 6 % sucrose all genotypes induced embryo Sagalato being the best, this was not determined as the best concentration since the embryos produced were white (instead of green) and the calli became brown on the 5th week. The browning of calli observed can be due to production of phenolic compounds and also osmotic stress (high osmotic pressure). Klenovska (1973) reported osmotic stress to have effects on the callus growth, shoot regeneration, somatic embryogenesis and metabolism of specific compounds. No big variations observed in response of landrace Rangimbili with varying sucrose concentrations while somatic embryo production in Kibandameno was highly reduced on 6 % sucrose. Sucrose has been normally used as a good source of carbon for regeneration in tissue culture. Naqvi et al. (2006) observed that a medium containing sucrose supported the callus proliferation and regeneration potential. Sucrose is strongly

metabolized making it available as an energy source for the plant to regenerate. Hence, MS media containing 2 % sucrose were used for subsequently somatic embryogenesis and regeneration of the selected cassava landraces. Four percent sucrose was assumed as optimum concentration for somatic embryo induction and regeneration to plantlets.

Similarly, Copper sulphate is reported to enhance primary embryo induction and decrease maturation time of somatic embryos (Danso and Ford-Lloyd, 2002; Nirwan and Kothari 2003). Since response to Copper sulphate is genotype dependent, the best somatic embryo responsive cassava landraces were tested using MS medium without and with addition of 1, 1.5, and 2 mM Copper sulphate. Using MS medium without additional Copper sulphate, only Kibandameno could produce an average of 2 embryos per explant used while the rest could not produce any embryo. However other unknown factors may have also contributed to the lack of somatic embryo production in the other landraces. Despite, all landraces produced somatic embryos at all concentrations of Copper sulphate in a varying rate. Purnhauser (1991) and Dahleen (1995) observed that higher levels of copper sulphate dramatically increased the regeneration ability of wheat and barley. Similar observation was from Nirwan and Kothari (2003) who reported an increase in callus formation and regeneration response of Sorghum bicolor (L), with increase in copper sulphate. The callusing induction and plant regeneration response was highly dependent on the concentration of copper sulphate in the MS medium having the optimal concentration at 2mM. Schöpke et al. (1992) reported that the regeneration of South American cassava cultivars was highly improved using MS medium with additional copper sulphate. Danso and Ford-Lloyd (2002) observed that copper sulphate improved callus induction, regeneration and also less maturation time of cassava somatic embryos. Similarly, Ihemere (2003) observed embryos undistinguishable from calli, produced on media lacking additional copper sulphate. The three somatic embryo responsive cassava landraces responded positively with an increase in copper sulphate concentration, 1.5 mM being the best concentration for Sagalato and Rangimbili. However, responses to additional copper sulphate were reported as genotype dependent (Zhang et al., 2000; Ihemere, 2003). Genotyping analysis of these three cassava landraces revealed them as relatively distantly related (Elibariki et al., 2013), suggesting that they are different genotypes and thus concurs with the literature.

Although light is known to enhance somatic embryogenesis and germination of embryos (Raemakers et al., 1997), in this study one experiment was conducted under limited light. The effect of exposing the explants into 16 hours photo period versus covering the plates with two layers of aluminum foil (limited light) under the same photoperiod was observed. Under limited light, all somatic embryogenic competent landraces could induce embryos in varying rates but the embryos were whitish as also observed by Ihemere (2003), suggesting lack of chlorophyll. Cassava landraces that showed less response to somatic embryogenesis ended up with calli of varying structures and size but no embryo could be produced. The callus turned brown and cells died on the 5^{th} week post inoculation. However, one cassava landrace (Gago) was observed to be highly responsive to somatic embryogenesis only under the limited light treatment. For this cassava landrace, all subsequently treatments to achieve somatic embryogenesis were through incubating the explants in the dark. However, all landraces tested under total darkness (plates placed in the dark incubator) induced calli but none of them could induce any embryo even when incubated beyond 12 weeks (results not shown).

In conclusion, 62 % Tanzanian farmer-preferred cassava landraces tested in this study demonstrated good ability in producing somatic embryos and regeneration potential. Response to somatic embryogenesis and regeneration ability was “cultivar” dependent as reported in the literature. Some of the landraces could be converted to plantlets while others could not. Taylor et al. (2001) also observed a similar phenomenon whereby 8 cassava genotypes failed to regenerate beyond the globular and torpedo stages. However, other factors like source and age of explants, culture conditions, sub-culturing cycles, age and brand of the media used might have contributed to the regeneration ability and variations of the tested cassava landraces in this work. Although only a few of these cassava landraces will be targeted for genetic engineering programs, results obtained from this study are enlightening potential candidate landraces amenable to transformation protocols. There is a need to develop efficient, genotype-independent regeneration and transformation protocols that will overcome a challenge of varying in vitro response of cassava between closely related cassava cultivars.

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Table 1 . Cassava landraces collected from major cassava growing zones in Tanzania and used in this study

Place of collection in Tanzania	Name of cassava landrace
Coast	Gago
	Karatasi
	Kibandameno
	Kibangili
	Mkongamwayo
	Mnazi
	Paja la Mzee
	Sagalato
South	Kaniki
	Kiroba
Lake Victoria Zone	Katakya
	Lyongonyeupe
	Milondikachini
	Rangimbili
	Rushura
Zanzibar Islands	Machui
	Mahonda
	Mwafaka
	Sepide
West	Shija

Table 2. Somatic embryogenesis of selected landraces under limited light

Cassava landrace	Number of embryos per total explants 5 weeks post-inoculation	Observations
Sagalato	40	All embryos were white
Rushura	0	White callus
Shija	0	Medium cream callus
Mwafaka	0	Medium cream callus
Kaniki	1	Big brown callus
Gago	9	Cream callus with whitish embryos
Gomani	0	Very big cream callus

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Table 3. Induction of somatic embryo cotyledons from Tanzanian farmer-preferred cassava landraces and subsequent regeneration to plantlets

Cassava landrace	Time (weeks) to induce embryo	Time (weeks) to reach cotyledonary stage	Regeneration stage achieved
Gago	6	8	plantlets
Kaniki	5	-	globular
Karatasi	-	-	callus
Katakya	5	7	plantlets
Kibandameno	5	6	plantlets
Kibangili	-	-	callus
Kiroba	5	-	globular
Lyongonyeupe	-	-	callus
Machui	6	8	globular
Mahonda	6	8	cotyledonary
Milondika chini	6	8	plantlets
Mkongamwayo	-	-	callus
Mnazi	4	6	plantlets
Mwafaka	-	-	callus
Paja la mzee	4	6	plantlets
Rangimbili	4	6	plantlets
Rushura	5	7	nary
Sagalato	4	6	plantlets
Sepide	-	-	callus
Shija	-	-	callus
Nachinyaya	-	-	callus

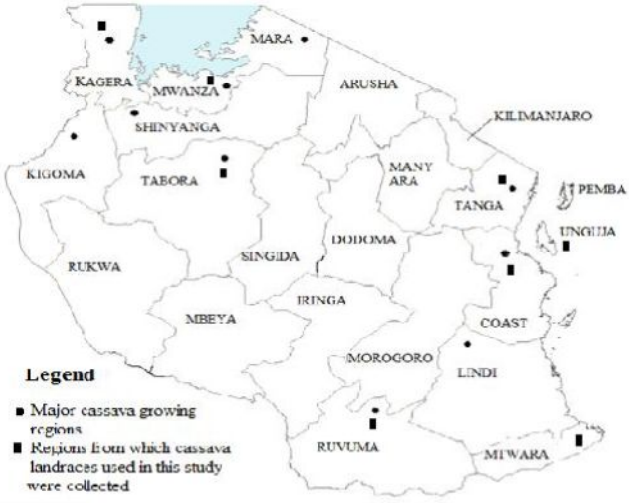


Figure 1. A map of Tanzania showing major cassava growing regions where samples used in this study were collected (Elibariki et al. 2013)

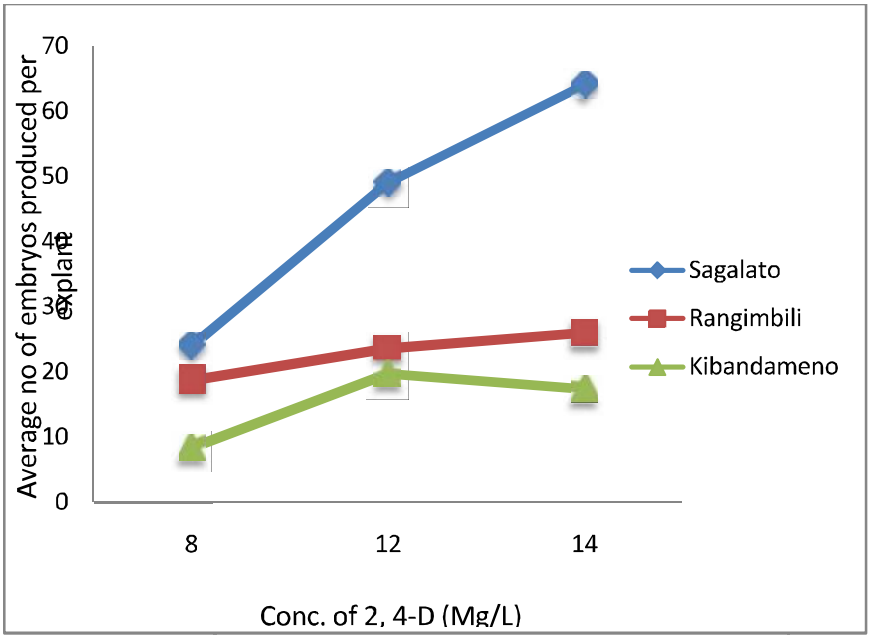


Figure 2. Somatic embryo response of selected cassava landraces on MS media with optimized 2, 4-D.

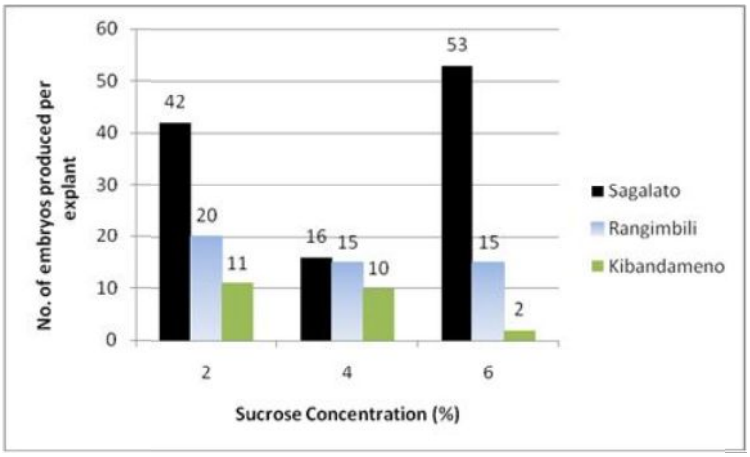


Figure 3. Somatic embryo response of selected cassava landraces on MS media with optimized sucrose.

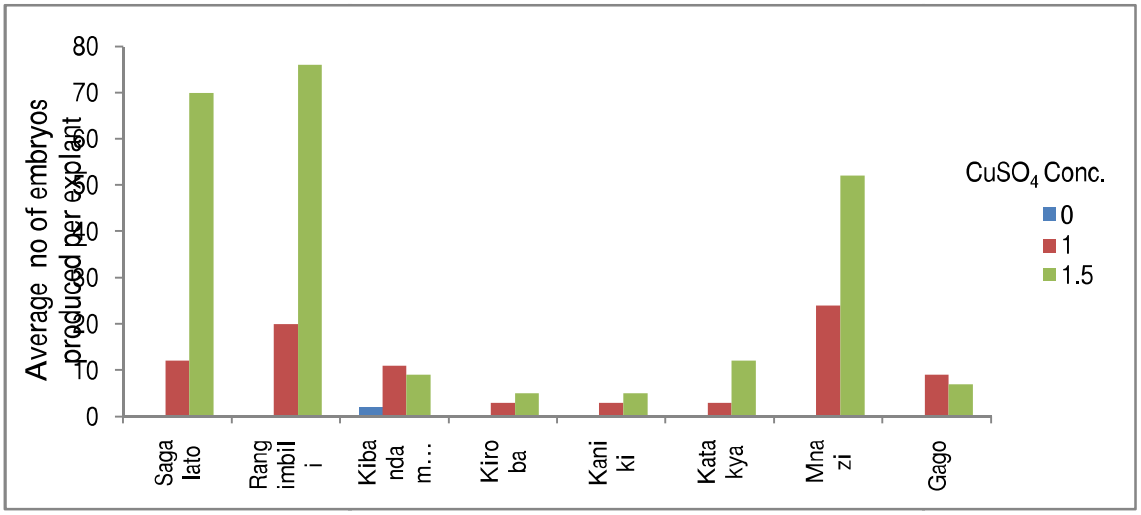


Figure 4. Somatic embryo response of selected cassava landraces on MS media with optimized Copper Sulphate

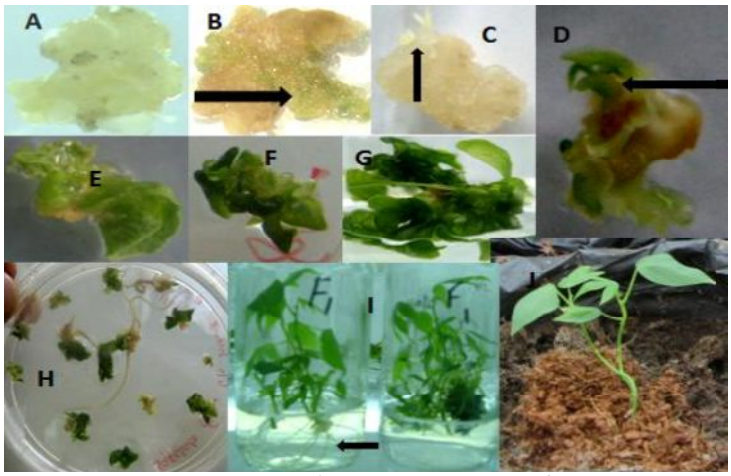


Figure 5. Somatic embryo induction and regeneration of a cassava landrace Sagalato landrace; A: callus arising from an explant; B: green embryoid structures arising from embryogenic callus C: embryos at torpedo stage; D: mature cotyledons; E, F and H: mature embryos on maturation media; G: multiple shoots arising from a single callus; I: Plantlets undergoing root maturation and J: 2 weeks plantlet on the soil

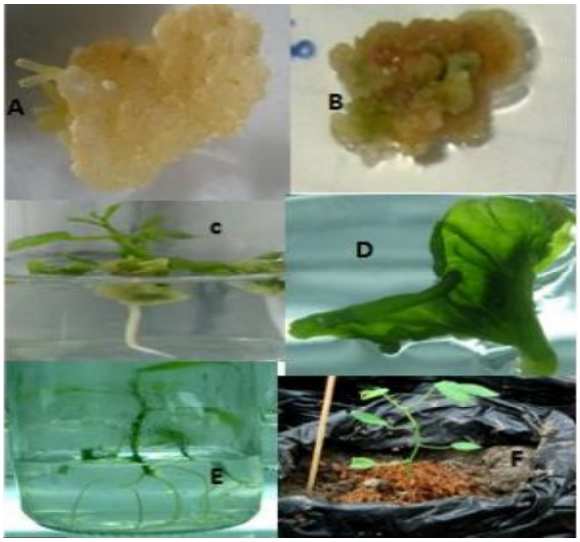


Figure 6 . Somatic embryo induction and regeneration of cassava landrace Mnazi. A: callus arising from an explant with torpedo stage embryos; B: green embryoid structures arising from embryogenic callus C: Mature cotyledons; D: Old cotyledon; E: a plantlet undergoing root maturation and F: Two weeks plantlet on the soil