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Efficient Plant Regeneration of Selected Kenyan Sweetpotato (*Ipomoea batatas* (L.) Lam.) Cultivars through Somatic Embryogenesis

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Abstract

Sweetpotato is an important food crop in the world as well as in Kenya. Various fungal and viral diseases are major constraints in its production and are currently threatening the sweetpotato production in sub-Saharan Africa. Genetic engineering offers significant potential for the crop's genetic improvement. However, this is limited by the low efficiency and strong genotype dependency in tissue culture. This study aimed to establish an efficient somatic embryogenesis and plant regeneration system using shoot apical meristem explants of sweetpotato. Three sweetpotato cultivars that are widely grown in Kenya; KSP36, Kemb36 and Mweu mutheke along with an exotic model cultivar Jewel were evaluated. The maximum somatic embryogenic induction, at 96.72%, was obtained from explants cultured on Linsmaier and Skoog salts and vitamins medium supplemented with 0.5 mg/l dichlorophenoxyacetic acid and 0.2 mg/l zeatin riboside. The highest number of shoot induction (33) was observed after transfer of embryonic callus to embryo maturation medium supplemented with 2 mg/l abscisic acid. Significant differences were observed between cultivars for somatic embryogenesis and plant regeneration. Jewel showed the best response, while Mweu mutheke was the least responsive under the culture conditions tested in this study. Regenerated plants were successfully rooted and grown to maturity after hardening in soil in the greenhouse. Such a robust, successful and efficient system possesses the potential to become an important tool for crop improvement and functional studies of genes in sweetpotato.

Keywords: Cultivars; Embryogenic callus; Plant regeneration; Somatic embryogenesis; Sweetpotato

Introduction

Sweetpotato, *Ipomoea batatas* (L.) Lam., which is a member of the family Convolvulaceae, is an important perennial crop. It ranks seventh in annual production among the food crops in the world. Its edible storage roots are not only a good source of energy due to carbohydrate content, but also used for starch and alcohol production [1-3]. It is mainly grown in the semi-arid tropical regions of Africa and Asia, for its edible tuberous roots, which are high in starch and vitamins, predominantly by small scale farming households operating at the margins of subsistence [4,5]. It is easily cultivated, has high production per unit area under both high and low input systems and requires fewer nutrients from the soil compared to most other starchy root and tuber crops [6].

Despite its many benefits, performance of sweetpotato yield (storage roots) is restricted to particular areas of the world due to agronomic practices and a number of unfavourable biotic and abiotic stresses such as viruses, fungi and nematodes and drought [6-8]. Almost all economically important traits including yield and yield components exhibit polygenic or quantitative inheritance [9]. The expression of quantitative traits is largely governed by environment in which they are exposed and thus, results into scale or rank shift of their performance [9-12]. Environment is defined as location × year combination. Year to year environmental variation is more unpredictable than location per se [10]. The complex genome of sweetpotato complicates environmental effects on selection of superior performing stable sweetpotato genotype across locations. Therefore, conventional breeding of sweetpotato is limited by the plant's complicated hexaploidy along with low seed production as a consequence of compatibility, sterility and special physiological requirements for flowering [12-14]. To overcome these limitations, efficient *in vitro* tissue culture and genetic engineering techniques are important biotechnological tools that can be used to complement breeding programmes for sweetpotato production. The success of plant regeneration, especially through somatic embryogenesis, relies primarily on synchronized production, maturation and conversion of embryos into plants [15]. Somatic embryogenesis therefore represents a promising tool of plant regeneration because embryogenic callus can be maintained for a long time and provides high multiplication rates [16,17].

Sweetpotato has long been considered recalcitrant in tissue culture particularly African sweetpotato cultivars [18,19]. Nevertheless, variety of protocols for sweetpotato regeneration and transformation of diverse sweetpotato cultivars have been been reported using different explants such as stems, petioles, leaves, storage roots, protoplasts and meristems. However, the low rate of embryo initiation, maturation, germination, and development into plantlets often remain a major challenge [20-29]. An efficient and practicable regeneration method of sweetpotato is still needed to meet the requirements of an effective genetic improvement of this crop. A high degree of genotype-

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dependent variation in embryogenic capacity has been witnessed, and remains a major impediment for developing transgenic sweetpotatoes [30-32]. To overcome these obstacles, a novel or modified somatic embryogenesis regeneration procedures must be established for each desirable cultivar, because of the significant variability in response to auxins and cytokinins combinations [33]. For these reasons, it is often important to develop a regeneration protocol that can either be used for specific or general sweetpotato cultivars. The present work reports a robust and reliable procedure for the establishment of an efficient and reproducible regeneration system via somatic embryogenesis for Kenyan sweetpotato cultivars using meristematic explants.

Materials and Methods

Plant material

Three farmer preferred Kenyan sweetpotato cultivars namely; KSP36, Kemb36, Mweu mutheke were used in this study. These cultivars were obtained from Kenya Agricultural and Livestock Research Organization gene bank at Muguga based on their wide adaptation in Kenya, high dry matter content and moderate to high resistance to sweetpotato virus diseases. Jewel (provided by the International Potato Centre, Nairobi), an America cultivar, which regenerates through embryogenesis and organogenesis [30,34] was included as the control. The selected cultivars were grown in a greenhouse at Plant Transformation Laboratory (PTL) at Kenyatta University, Nairobi. These stock plants were used to supply vines for initiation of *in vitro* cultures.

In vitro culture initiation

Stems containing the shoot apex and 2 to 3 lateral buds were collected from plants grown in greenhouse. All branches and leaves were cut off and the vines were thoroughly washed in running tap water to remove dirt. The vines were surface sterilized with solution containing 50% v/v commercial JIK (Reckitt Benckiser, Nairobi, Kenya) and 0.01% v/v of Tween 20 (Sigma-Aldrich, St. Louis, USA) for 20 min. After surface sterilization, the vines were transferred to sterile water and rinsed three times. Apical meristems were carefully excised from the vines and placed onto sweetpotato propagation medium (SPM) in 70 mm diameter glass culture bottles. The culture medium was made by mixing Linsmaier and Skoog (1965) salts and vitamins (LS) supplemented with 30 g/l sucrose and the pH of the medium was adjusted to 5.8 before 8 g/l agar was added followed by autoclaving at 121°C for 15 min under 15 kPa. *In vitro* sweetpotato plants were grown at a temperature of 27 ± 1°C, 16/8 h (light/dark) photoperiod provided by white fluorescent lamps and 70% relative humidity. The *in vitro* plants were subcultured by cultivating node sections on a monthly basis and the sterile plants were used as the source of the explants for somatic embryogenesis.

Callus induction

Stem sections from three to four week old *in vitro* stock plants were transversely cut into 6-10 mm sections. These sections were then cut in half along the axis and used as explants for callus induction. For callus induction, the explants were transferred onto callus induction medium (CIM), keeping the cut surface in contact with the medium containing LS salts and vitamins supplemented with 30 g/l sucrose, 8 g/l agar, 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) for 5-6 days in growth room at a temperature of 27 ± 1°C, 16/8 h (light/dark) photoperiod provided by white fluorescent lamps and 70% relative humidity. After 5-6 days on CIM, the explants were transferred to medium that contains LS salts and vitamins supplemented with 30 g/l sucrose, 0.2 mg/l zeatin riboside (ZR) and 8 g/l agar (CPM) to promote the formation of callus. The explants were subcultured to a fresh media after every two weeks until they became yellowish.

Somatic embryo development and plant regeneration

After 4 weeks, calli that formed were transferred onto embryo induction medium (EIM) containing LS salts and vitamins, 30 g/l sucrose, 0.5 mg/l 2,4-D, 0.1 mg/l gibberellic acid (GA_3) and 8 g/l agar. The calli were subcultured onto fresh EIM medium after every two weeks until differentiation into embryo-like structures were observed. These embryos were then transferred to embryo maturation medium (EMM) containing LS salts and vitamins, 30 g/l sucrose, 2 mg/l abscisic acid (ABA) and 8 g/l agar. The regenerated shoots were transferred to SP propagation medium and maintained at a temperature of 27 ± 1°C, 16/8 h (light/dark) photoperiod provided by white fluorescent lamps and 70% relative humidity. Regeneration frequencies were recorded after four weeks in culture.

Data analysis

Data on effects of cultivar on germination on LS medium, effects of 2,4-D and ZR on callus induction, effects of 2,4-D and GA_3 on somatic embryogenesis and germination and regeneration frequencies were analysed using ANOVA with Minitab statistical computer software v.17 (Minitab Inc., Pennsylvania, U.S.A). Means were separated using Tukey's Honest Significant Difference test at a confidence level of 95% ($p \leq 0.05$).

Results

In vitro culture initiation

In vitro culture stocks of Jewel, Kemb36, KSP36 and Mweu mutheke cultivars were established using apical shoot meristem culture. It was possible to establish *in vitro* plants of all selected cultivars using the LS medium, albeit with different efficiencies. Kemb36 exhibited the highest plant formation frequency of 92.09% followed by Mweu mutheke and KSP36 with plant formation frequencies of 86.67% and 83.09%, respectively. Jewel had the least plant formation frequency of 68.27% (Table 1). There was significant difference between Jewel and Kemb36 in terms of plant regeneration efficiency on LS medium (Table 1, $p<0.05$). However, for cultivars Kemb36, KSP36 and Mweu mutheke, there was no significant difference, in terms of plant regeneration efficiency in LS medium (Table 1, $p>0.05$). All plants formed from apical meristems exhibited normal phenotype.

Callus induction

All the sweetpotato cultivars in the media supplemented with 2,4-D and ZR. For callus induction I and II, Jewel, Kemb36, KSP36 and Mweu mutheke on CIM and CPM media produced 48.00, 49.00, 50.33 and 58.33 average calli, respectively. There was no significant difference in

Cultivar	No. of meristems	No. of plants regenerated[x]	Regeneration efficiency[y]
Jewel	58	13.33 ± 4.16[a]	68.27 ± 6.08[a]
Kemb36	57	17.33 ± 0.58[a]	92.09 ± 9.32[b]
KSP36	51	14.00 ± 4.36[a]	83.09 ± 8.34[ab]
Mweu mutheke	47	13.67 ± 4.04[a]	86.67 ± 3.33[ab]

All meristems were cultured in SPM. [x]Mean ± standard deviation. Means are from three replicates. [y]Regeneration efficiencies were calculated as the percentage number of apical meristems that regenerated into plants from the total number of starting apical meristems. Values followed by different letters in the same column are significantly different ($p<0.05$) according to Tukey's Honest Significant Difference test

Table 1: Sweetpotato cultivar variation on *in vitro* culture initiation.

callus induction response in the medium supplemented with 2,4-D and ZR among the sweetpotato cultivars (Table 2). Callus induction began as swollen regions at the cut ends of the explants. The non-embryogenic and pro-embryonic masses with tightly packed isodiametric cells developed from the stem explants or calli are presented in Figures 1A and 1B, respectively.

Somatic embryo development and plant regeneration

Four months after the transfer of calli onto the EIM, somatic embryogenic structures developed. All sweetpotato cultivars produced somatic embryos on embryogenic calli formed. Jewel and KSP36 were the best responding cultivars with regards to somatic embryogenesis on EIM ($p<0.05$, Table 3) with embryogenic calli formation frequencies of 96.72% and 89.56, respectively while Kemb36 and Mweu mutheke produced the lowest somatic embryos with embryogenic calli formation frequencies of 76.63 % and 60.86%, respectively (Table 3). Jewel and KSP36 showed no significant difference in somatic embryogenic callus formation frequency ($p>0.05$, Table 3). However, there was significant difference in somatic embryogenic callus formation frequency between Kemb36 and Mweu mutheke ($p<0.05$, Table 3). Callus from Jewel explants grew better and turned yellow faster compared to calli from KSP36 explants. The growth and somatic embryogenic response of calli from Kemb36 and Mweu mutheke was the least. Somatic embryogenesis began at swollen regions at the cut ends giving rise to three different types of calli: non-embryogenic calli (Figure 1A), embryogenic calli (Figure 1B) and some with embryogenic and non-embryogenic sections on the same callus.

The greenish compact, and slow growing calli that developed organized structures and eventually exhibited tissue differentiation were regarded as embryogenic (Figure 1C), in contrast to the non-embryogenic calli that were white or cream, friable and fast growing. After 4-5 weeks on EIM medium, some calli of the sweetpotato cultivars turned green and formed globular, heart, torpedo and cotyledonary stages of embryos which appeared successively. Although somatic embryogenesis callus formation frequencies were significantly high across all the cultivars, the regeneration frequencies were modest ranging from 22.94% for Mweu mutheke to 71.22% for Jewel (Table 3).

Cultivar	No. of Explants	Callus induction I[x]	Callus induction II[x]
Jewel	144	48.00 ± 10.54[a]	48.00 ± 10.54[a]
Kemb36	147	49.00 ± 8.19[a]	49.00 ± 8.19[a]
KSP36	151	50.33 ± 13.58[a]	50.33 ± 13.58[a]
Mweu mutheke	175	58.33 ± 11.93[a]	58.33 ± 11.93[a]

[x]Values indicate the average numbers of calli per explant cultured on CIM and CPM media (Mean ± standard deviation). Means are from three replicates. Values followed by same letter in the same column are not significantly different ($p>0.05$) according to Tukey's Honest Significant Difference test

Table 2: Effects of 2,4-D and ZR on callus induction on sweetpotato cultivars.

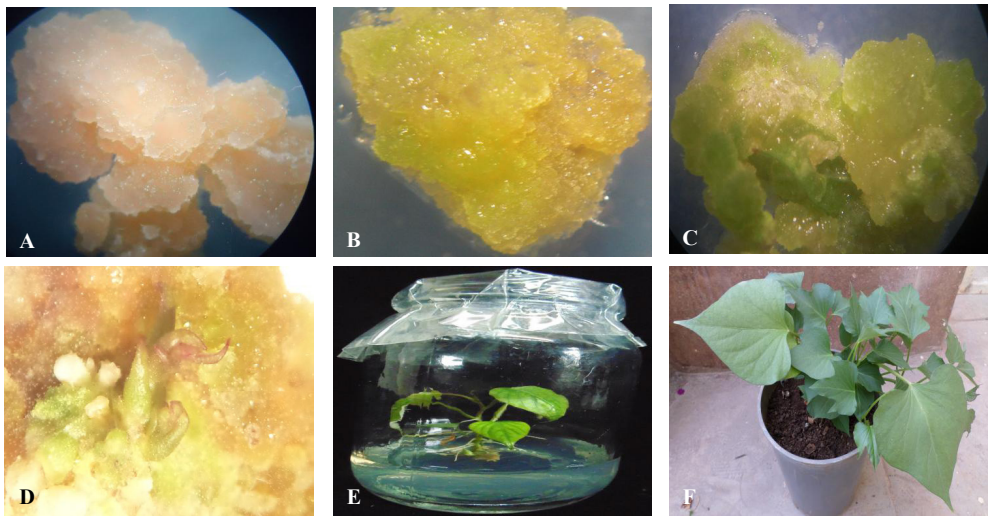

Figure 1: Sweetpotato tissue culture regeneration profile via somatic embryogenesis. **A.** white friable non-embryogenic callus; **B** and **C.** Greening of embryogenic calli and shoot development on **EIM**; **D.** Regenerating tissue on EMM. Callus; **E.** Regenerated plant on SPM; **F.** Mature plant in greenhouse.

Cultivar	Initial No. of Explant	No. of embryogenic calli[x]	Embryogenic calli regeneration frequency[y]	No. of shoots regenerated[z]	Regeneration frequency[z]
Jewel	144	46.33 ± 9.50[a]	96.72 ± 1.58[c]	33.33 ± 10.21[c]	71.22 ± 8.70[c]
Kemb36	147	37.33 ± 5.13[a]	76.63 ± 5.96[b]	15.33 ± 0.58[ab]	41.44 ± 4.13[b]
KSP36	151	45.00 ± 12.00[a]	89.56 ± 2.69[c]	29.00 ± 8.89[bc]	64.29 ± 5.71[c]
Mweu mutheke	175	35.67 ± 8.39[a]	60.86 ± 3.41[a]	8.00 ± 1.73[a]	22.94 ± 4.88[a]

[x]The values (Mean ± standard deviation) indicate the average numbers of calli cultured on EIM media. [y]Embryogenic calli formation efficiency was calculated as the percentage number of meristems that formed embryogenic calli from the total number of starting meristems. [z]Regeneration frequency was calculated as the percentage number of embryogenic calli that regenerated shoots from the total number of starting embryogenic calli. Values followed by same letter in the same column are not significantly different ($p>0.05$) according to Tukey's Honest Significant Difference test

Table 3: Somatic embryogenesis and regeneration of different sweetpotato cultivars.

Embryonic structures developed after 4 months of culture and mature somatic embryos that were formed were transferred onto embryo maturation medium (EMM) supplemented with 2 mg/l abscisic acid medium. After two months on EMM medium, shoot-like structures emerged from the callus (Figure 1D). A few of the calli differentiated to form embryonic structures and a fewer embryos ultimately produced plants. After approximately 8 weeks, shoots that initially had an unusual appearance (thickened) reverted to a normal phenotype, and were transferred onto SPM for plant propagation (Figure 1E). The *in vitro* regenerated plants were hardened grew normally in the greenhouse successfully (Figure 1F).

Discussion

In this study, *in vitro* plants from four selected sweetpotato cultivars were established by meristem culture and used to obtain stem explants for the successful induction of embryogenic tissues and subsequent plant regeneration. Variations in shoot-regeneration among sweetpotato cultivars observed in this study were reported in previous studies for sweetpotato when shoot tips were used as the source of explants [16,20,35]. This implies that germination of sweetpotato apical meristems is cultivar-dependent.

Plant regeneration through somatic embryogenesis largely relies on synchronized production, maturation and conversion of embryos into plants [15]. The phenomenon of somatic embryogenesis requires attainment of embryogenic competence via dedifferentiation, chromatin remodelling and gene expression programming in somatic cells [36,37]. Somatic embryogenesis procedures are preferred over shoot organogenesis because somatic embryogenesis is the most efficient method for regenerating genetically transformed sweetpotato plants, although this technique is constrained by low regeneration efficiency and strong effects of cultivar-dependency. In order to induce somatic embryogenesis in sweetpotato, a two-stage approach, by first exposing explants to 2,4-D for 5 to 6 days, followed by a prolonged exposure to ZR in the second stage was found to be the most effective. The induction of callus in sweetpotato is commonly achieved when the medium is supplemented with 2,4-D [16,23,38]. However, in this study, addition of ZR to medium containing 0.5 mg/l 2,4-D increased the percentage of somatic embryogenic induction from shoot apex explants. Gosukonda et al. [39] also used the two-stage approach for somatic embryogenesis in sweetpotato. A similar technique, where the auxin 0.5 mg/l 2,4-D was replaced by 4-fluorophenoxyacetic acid in the first stage followed by a prolonged exposure to ZR, was reported for sweetpotato Jewel, yielding 100% shoot regeneration of stably transformed transgenic plants [30]. In this study, 0.5 mg/l 2,4-D was used in the first stage and was found to be effective for promoting callus induction when explants were exposed for 5 to 6 days in callus induction medium. Dessai et al. [40] and Gosukonda et al. [39] previously observed that a slightly longer exposure to auxin in the first stage can cause profuse callusing at the end of petioles and failure to regenerate in the subsequent stages. It is therefore, plausible that the 5 to 6 days exposure to 2,4-D in the initial stage in this study may have been too long. While this may be the case, this study established that 5 to 6 days were necessary for swelling of the stem to occur, which is a requirement for the transition to the second-stage hormone treatment. Some explants developed multiple adventitious shoots that emerged from callus at the cut site of petioles, in accordance to previous observations by Sefasi et al. [18] and Santa-Maria et al. [41]. The effect of lower or higher ZR concentrations for promoting either rooting or compact callus development has also been reported in earlier study by Dessai et al. [40] who observed that induced adventitious shoots formation. Therefore, to achieve efficient shoot regeneration in sweetpotato, a brief exposure of explants to 2,4-D and followed by a cytokinin may be very critical. This phenomenon may be due to explants acquiring 'competency' for regeneration after a specific period of exposure to an auxin at which stage they would be most responsive to the cytokinin thus resulting in high shoot regeneration.

Typical developmental phases were observed in somatic embryos developing from both callus-mediated and those directly arising from explants. Pro-embryonic masses with tightly packed isodiametric cells developed from the stem explants or calli from which the globular, heart, torpedo and cotyledonary stages appeared successively. Somatic embryos appeared distinctively green against the yellowish background of the callus or the explant. Results of this study demonstrate that stem explants can be readily regenerated to form shoots. Stem explants derived from meristem culture are excellent source material over other explants such as apical meristems due to easy storage and accessibility to large amounts of uniform quality explant material explant for sweetpotato regeneration [28].

Maturation of somatic embryos involves the development of globular embryos into cotyledonary embryos with defined shoot and root axes [42]. The use of ABA was vital for the maturation of sweetpotato somatic embryos, resulting in the development of morphologically normal plants. The mode of action of ABA during embryo development is not well understood, however, it is commonly employed to synchronise somatic embryogenesis and improve developmental attributes and conversion of somatic embryos to shoots [43,44]. Prolonged exposure of somatic embryos to ABA possibly stimulates embryo development and subsequent germination and conversion of the embryos to shoots [45].

Findings from the present study show that regeneration of the sweetpotato cultivars was genotype dependent. Therefore, the genetic structure appeared to be the most critical factor governing varying degree of tissue culture response of cultivars. The major influence on tissue culture response appears to be genetic, with tissue culture requirements varying between cultivars. Consequently, the regenerative capability of various explants is dependent on sweetpotato cultivar. In addition to cultivar-dependent response, the presence of meristematic tissues in young stems may have contributed to higher regeneration frequencies. Meristem tissues contain actively dividing cells that are responsible for length extension of the plant body and therefore have a greater capacity for regeneration [32]. Screening of varieties for their response in tissue culture is important since it allows the identification of genotypes that are more amenable for manipulation. In this study, the four sweetpotato cultivars tested were able to undergo somatic embryogenesis and plant regeneration under the culture conditions tested. However, the degree of morphogenic response was found to be cultivar-dependent. Jewel was the most responsive in all treatments, while Mweu mutheke was consistently the least responsive.

In conclusion, a high-throughput system for somatic embryogenesis and plant regeneration has been developed which is effective across four sweetpotato cultivars. This was attained by combining auxin and cytokinin growth regulators at appropriate combinations and concentrations in order to stimulate callus induction, somatic embryogenesis and plant regenerations from meristem explants. This protocol serves as a basis for transgenic technologies or genome editing for this important crop.

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