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In Vitro Plant Regeneration of Sweetpotato Through Direct Shoot Organogenesis

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Abstract

Sweetpotato (*Ipomoea batata*) is an important, nutritionally rich vegetable crop, but severely affected by environmental stresses, pests and diseases which cause massive yield and quality losses. Genetic manipulation is becoming an important method for sweetpotato improvement. In the present study, a reproducible and highly efficient protocol for *in vitro* plant regeneration of six Kenyan farmer preferred sweetpotato, Enaironi, KEMB 36, KSP36, Mugande, Kalamb Nyerere, SPK 013 and SPK004 through direct shoot organogenesis from stem internodes explants was developed. The results revealed that *Kalamb nyerere* had the highest number of adventitious bud; for light (5.33 and 4.33) and dark (8.00 and 5.00) induction condition for all TDZ hormone level (0.25 mg/l and 0.15 mg/l). When explants incubated in 0.10 mg/l NAA the regeneration frequencies were the highest at 83.33% (*Jewel)* and 96.67% (*Kalamb nyerere*) for adventitious buds recovered from light and darkness respectively. This was the optimal auxin concentration which gave the maximum regeneration frequency with adventitious buds recovered from the dark. The best Kenyan sweetpotato genotypes for direct shoot organogenesis were *Kalamb nyerere, Kemb 36* and *SPK 004*. The protocol presented in this work is suitable for improvement of sweetpotato genotypes through tissue culture methods and or genetic transformation.

Keywords: Adventitious buds; Organogenesis; Regeneration; Sweetpotato; TDZ

Introduction

In vitro organogenesis is a process that leads to organ formation through dedifferentiation of differentiated cells into callus and reorganization of cell division to form specific organ primordia and meristems [1]. In direct shoot organogenesis, the shoots are formed directly without a callus intermediate stage. Such a process, adventitious bud formation, is attractive for plant regeneration and improvement since it has the advantage of being quick and or faster with minimal somaclonal and chimeric variations [2]. Induction signal is a requirement for any plant organogenic regeneration process. This being the case, two distinct organogenic induction signals has been reported for direct shoot organogenesis or adventitious bud formation. One signal is required to induce shoots and the other to induce roots. Organogenic callus induction from explants requires a high auxin to cytokinin ratio in the media whereas shoot formation is triggered by a high cytokinin to auxin ratio or high cytokinin with no auxin [3]. Such shoots originate from unipolar and callus-derived organ primordia that form roots upon transfer to medium without hormones or containing natural rooting auxins such as isobutyric acid (IBA) and α-naphthalene acetic acid (NAA) [2].

Thidiazuron (TDZ), is a unique growth regulator that structurally has two functional groups; a phenyl and thidiazol groups with complimentary roles in activity [4]. Exposure of explants to low concentration of TDZ either alone or in combination with other growth-regulating substances in the induction media for short periods of time is enough to induce adventitious bud [5]. Indeed, TDZ alone can be applied as a substitute for both the auxin and cytokinin requirement in many species [6].

Sweetpotato (*Ipomoea batatas* Lam*)* is a domesticated open pollinated, self-incompatible hexaploid, whose basic chromosome number x=15 while the wild *Ipomoea* species can be diploid, tetraploid or hexaploid. It is an autopolyploid showing polysomic inheritance and self-sterility thus propagated vegetatively. Adventitious shoot formation as a method therefore can be used for overcoming cross-ability reproductive barriers caused by scarce natural flowering saprophytic incompatibility and partial male/female sterility facing *Ipomoea* landraces as well maintaining the clonal fidelity of sweetpotato. Different explants have been used for organogenic callus cultures with subsequent shoot organogenesis such as tobacco (*Nicotiana tabacum* L.) protoplasts, *Arabidopsis thaliana* root and leaf explants [7], strawberry shoot organogenesis using leaf disks and petioles [8]. However, sweetpotato regeneration through adventitious bud formation at a high frequency has been restricted to a few exotic genotypes since a wide range of African cultivars are recalcitrant or respond at low frequencies [9]. Besides most of the reported regeneration protocols that have worked with exotic varieties in some laboratories are difficult to reproduce in others particularly when recalcitrant African sweetpotato varieties are used thereby further compounding the problem [10]. Indeed, it has been reported that different media formulation and type of plant organ providing the explants have been shown to influence *in vitro* regeneration [9,11]. The objective of this study was to develop an efficient reproducible protocol of plant regeneration for recalcitrant Kenyan sweetpotato genotypes that can be used for subsequent improvement of the sweetpotato genotypes through genetic transformation such as to enhance salt tolerance.

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Materials and Methods

Plant material and culture conditions

Six selected Kenyan sweetpotato genotypes (*SPK004, KSP36, KEMB 36, SPK 013, Enaironi, Mugande and Kalamb Nyerere*) were obtained from Kenya Agriculture and Livestock Research Organization biotechnology center GeneBank. A model variety Jewel was obtained from Potato Improvement Centre, Nairobi. Vigorously growing vines from green house plants were used to provide cuttings for establishment of *in vitro* stock cultures. The cuttings were surface sterilized by immersing them in commercial Jik containing 3.85% NaOCl and two drops of Tween 20 for 10 minutes before transferring to 70% ethanol for further disinfection in the laminar flow chamber for a period of 5 minutes. Thereafter, the cuttings were rinsed three times in sterile double distilled water [11].

The sterile cuttings were incubated into Sweetpotato Propagation (SP) medium in culture bottles in a medium composed of MS [12] salts supplemented with sucrose (30 g/l), myo-inositol (0.1 g/l), indole-3 acetic acid (IAA) (1.0 µM), 5 ml/l sweetpotato vitamin stock comprising 40 g/l ascorbic acid, 20 g/l L-arginine, 4 g/l putrescine HCl, 5.8 µM gibberellic acid (GA_3) and 0.4 g/l calcium pantothenate. The medium pH was adjusted to 5.8 before adding 3 g/L gelrite, followed by autoclaving at 121°C for 15 minutes under 15 kPa.

Adventitious bud induction on TDZ medium

Bud induction medium was composed of MS basal salts supplemented with sucrose (30 g/l), myo-inositol (0.1 g/l) and sweetpotato vitamin stock. Since bud induction experiments using most recalcitrant species are done at low Thidiazuron (TDZ) concentration, two concentrations of Thidiazuron, 0.15 mg/l and 0.25 mg/l, were added to separate media after autoclaving based on a protocol by previous works [3] with slight modification. Stem internodes segments (0.6-1.0 cm) were cut from 4-week-old *in vitro* cultures growing in SP medium in culture bottles. The explants were horizontally placed and partially pressed into the medium and cultured on 25 ml of induction medium in plastic petri-dishes. The petri-dishes containing the cultures were placed in the dark for 7 days to induce adventitious buds before transfer to light for shoot regeneration for 4 weeks. The cultures were then transferred onto fresh regeneration media after the induction period.

Effect of light on adventitious bud induction on TDZ medium

To assess the effect of light on bud induction of explants on medium containing TDZ, half of the explants were placed on the medium with TDZ for 7 days in the dark at 28°C, while the other half was incubated in 16 hours of cool white light (3000 lm) and about 180 µmol/m^2/s light intensity for the same period. Afterwards, the petri-dishes containing the cultures were transferred to light for shoot regeneration for 4 weeks as previously described.

Adventitious bud regeneration on auxin containing medium

Due to low frequency of conversion of adventitious buds into shoots, the effect of α-naphthalene acetic acid (NAA) on conversion of adventitious buds into shoots was also investigated. The adventitious buds induced in both light and dark as previously described were used. Thereafter, each group of explants was then further divided into three equal groups; one group was transferred to medium that did not contain any NAA while the other groups were transferred to media containing 0.1 mg/l NAA and 0.25 mg/l NAA respectively, before incubating them for 16 hours of cool white light (3000 lm) and about 180 µmol/m^2/s light intensity at 26°C temperature, for shoot regeneration.

Data analysis

All experiments were carried out in a completely randomized design. Three petri-dishes, containing 10 explants each, were used in each experiment that was replicated three times. Data of the number of buds per explants were recorded after 4 weeks in culture. Similarly, data on the number of explants with adventitious shoots, with developed leaves and roots and number of adventitious shoots per explants were collected after 12 weeks of culture. The frequency of explants regenerating adventitious buds and shoots was calculated by expressing the number of explants regenerating buds or shoots as a percent of the total number of explants investigated. Statistical analyses were done using analysis of variance with SAS computer software (version 9.1.3; SAS Institute Inc. Cary, NC). Means were separated using Least Significant Difference test at a confidence level of 95% ($p \leq 0.05$).

Results and Discussion

Adventitious bud induction frequencies

When explants obtained from *in vitro* grown plants were incubated in induction media containing two different concentration of TDZ, they swelled and their colour changed within 3 days. The explants incubated in the dark tended to be pale yellow while those in light were light green in colour (Figure 1a). At the end of induction period, adventitious buds were observed on the explant surfaces. By day 11 of culture, a few small white callus-like protuberances were formed in the cut regions in all the regenerated genotypes (Figure 1a). Induction frequencies in light revealed that *Kalamb nyerere* (5.33) and (4.33) was significantly high in 0.25 mg/l and 0.15 mg/l TDZ levels. Similarly, *Kalamb nyerere* had highly significant frequencies (8.00) and (5.00) respectively at the two-hormonal level on average when explants were incubated in the dark ($p \leq 0.05$; Table 1).

After a few days of culture, cells increased in size and then started initial division around the vascular bundles which may explain why callus-like protuberances were observed on the stem explants. The variable bud induction frequency observed when using stem explants was genotype dependent. The cells of the explants in the presence of TDZ, a cytokinin, alone were able to acquire competence, dedifferentiate and redifferentiate to form adventitious buds that later were able to form entire plants in regeneration media. This observation is in agreement with [3] working with Ugandan sweetpotato genotypes which showed that cytokinin level and the genotype affected the induced adventitious buds using TDZ. Other types of explants have been used for bud induction with varying degree of success such as root, leaves, nodal segments, auxiliary buds and even leaf stalks [3,13]. In the present study, it was observed that the highest induction frequencies of adventitious but was at low TDZ concentration (0.25 mg/l) however lower concentrations recorded lower numbers of bud induction. Similar effects were previously investigated by several authors who used lower levels of cytokinin to induce adventitious buds [3,13,14].

Results indicate that when the explants were incubated in the dark the number of adventitious bud induced were higher than those recovered from light. Similar findings have been reported by another researcher. For example, Previous studies observed 50% frequency increase of *Phaseolus angularis* L. regenerating buds when explants were pre-cultured in the dark [14]. Such observation indicates that for high bud induction frequencies incubation in the dark than light is the prefered condition for all genotypes tested. It is safe to conclude that dark conditions are a prerequisite to maximal induction of the initial morphogenetic potential in cells within the genotypes. This observation

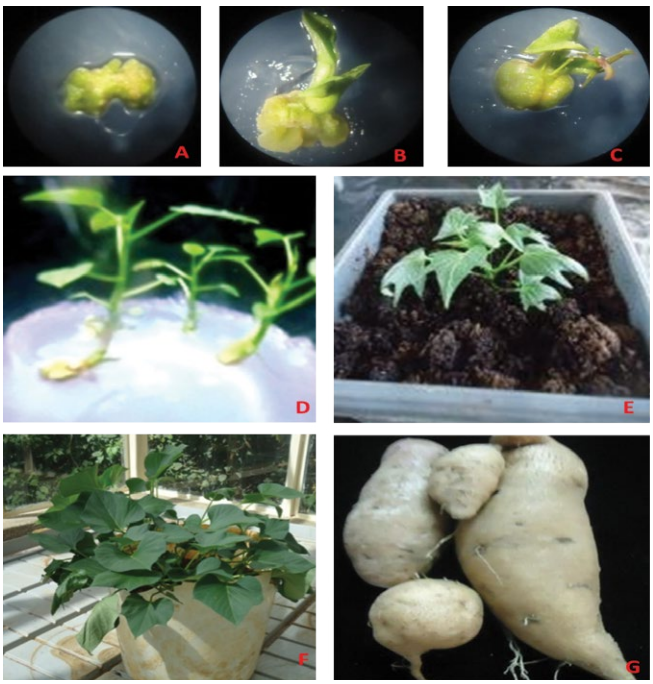

Figure 1: Regeneration of Sweet potato genotypes through organogenesis. A. Adventitious bud forming after induction in TDZ hormone in the dark. B. Germinating bud forming plantlet surrounded by callus tissue. C. Plantlets in regeneration media. D. Plants in shooting media. E. *Kalamb Nyere* plants in peat moss during hardening. F. *Jewel* sweet potato plants in the glass house. G. Harvest from mature jewel plant after four months.

	TDZ level (Dark)		TDZ level (Light)
Genotype/hormone	0.15 mg	0.25 mg	0.15 mg
Enaironi	3.00 ± 0.41^{c}	5.00 ± 0.41^{cd}	2.00 ± 0.50^{bc}
Jewel	5.00 ± 0.41^{ab}	7.00 ± 0.41^{ab}	3.67 ± 0.33^{ab}
K. Nyerere	6.00 ± 0.41^{a}	8.00 ± 0.41^{a}	4.33 ± 0.44^{a}
KSP 36	4.00 ± 0.41^{bc}	5.67 ± 0.33^{bcd}	3.00 ± 0.41^{abc}
Kemb 36	5.33 ± 0.33^{ab}	7.00 ± 0.41^{ab}	3.33 ± 0.41^{ab}
Mugande	3.67 ± 0.53^{bc}	4.33 ± 0.44^{d}	1.33 ± 0.33^{c}
SPK 004	5.00 ± 0.41^{ab}	6.33 ± 0.33^{ab}	2.00 ± 0.41^{bc}
LSD	1.81	1.7	1.77

Values are means and standard errors of 3 replicates with 10 explants per petri plate replicated 3 times within genotypes for each treatment. Means followed by the same letter in each column are not significantly different from each other (Tukey's test; $p \leq 0.05$).

Table 1: Adventitious bud induction in seven sweet potato genotypes using Thidiazuron hormone (TDZ).

has been reported to involve the PhyB genes as observed in Arabidopsis [15].

Structurally, there are two functional groups in a TDZ molecule; phenyl (adenine type) and thidiazol groups that have complimentary roles in TDZ-induced responses [4]. Reports indicate that adenine and phenylurea type cytokinins have a common binding receptor in the plant cell, a cytokinin - binding protein (CBP) with two different binding sites at the molecular level. One site binds adenine-type cytokinins naturally, while the other is able to bind phenylurea-type cytokinins [16]. Binding of an adenine-type cytokinin to CBP induces the expression of the rapid cytokinin genes, IBC6 and IBC7, thereby promoting cell division as well as stimulating tissue and shoot formation [17].

Other reports indicate that when TDZ is exogenously applied, the phenylurea CBP site becomes occupied that enhances the effect of exogenous and or endogenous adenine - type cytokinin already bound to CBP. Alternatively, TDZ promote growth due to its own biological cytokinin activity and also induces the synthesis and or accumulation of endogenous cytokinins [18]. Additionally, TDZ also enhances the availability and accumulation of endogenous cytokinins through non-competitively inhibition of cytokinin oxidase activity [19].

Regeneration frequencies in NAA media

When the explants were transferred to regeneration media, not all the adventitious buds induced were converted to plantlets. The efficiency of conversion was dependent largely on the concentration of NAA in the regeneration media and the genotype. From this study only two genotypes (*Kalamb nyerere*) and (*SPK 004*) in light and dark induction respectively were able to regenerate significantly ($p \leq 0.05$) in hormone free regeneration media. Most of the genotypes showed adventitious bud abortion, irrespective of the induction source and photoperiod in hormone free media (Tables 2 and 3). Regeneration media supplemented with 0.1 mg/l NAA had significantly ($p \leq 0.05$) highest adventitious bud conversions to plantlets (*Kalamb nyerere*, 83.33 and 96.67) whether the buds were induced in light or darkness. Additionally, adventitious bud conversion to plantlets showed significantly ($p \leq 0.05$) variable regeneration percentages for all genotypes when buds were recovered in light or darkness (Tables 2 and 3). Interestingly, the results from 0.25 mg/l NAA level showed only one genotype (*Kalamb nyerere*) with significantly ($p \leq 0.05$) different percentage plant regeneration while the other varieties suffered adventitious bud abortions in light. Adventitious bud induced in the dark had three genotypes (*Enaironi, Jewel* and *Kalamb nyerere*) that regenerated significantly ($p \leq 0.05$) while the rest suffered bud abortions (Table 3).

The adventitious bud regeneration frequency in the present study was

Genotype/	Light			Dark		
NAA(mg/l)	0	0.1	0.25	0	0.1	0.25
Enaironi	0.00 ± 0.00^{b}	20.00 ± 3.84^{bc}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	46.67 ± 1.89^{b}	20.00 ± 3.43^{a}
Jewel	0.00 ± 0.00^{b}	20.00 ± 2.76^{bc}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	33.33 ± 2.77^{bc}	26.67 ± 3.10^{a}
K. Nyerere	20.00 ± 4.23^{a}	83.33 ± 3.84^{a}	50.00 ± 4.33^{a}	0.00 ± 0.00^{b}	96.67 ± 4.62^{a}	26.67 ± 3.23^{a}
KSP36	0.00 ± 0.00^{b}	0.00 ± 0.00^{d}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	20.00 ± 4.50^{cd}	0.00 ± 0.00^{b}
Kemb36	0.00 ± 0.00^{b}	16.67 ± 2.86^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	46.67 ± 3.48^{b}	0.00 ± 0.00^{b}
Mugande	0.00 ± 0.00^{b}	0.00 ± 0.00^{d}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	16.67 ± 2.89^{d}	0.00 ± 0.00^{b}
SPK004	0.00 ± 0.00^{b}	33.33 ± 1.65^{b}	0.00 ± 0.00^{b}	20.00 ± 4.63^{a}	46.67 ± 0.63^{b}	0.00 ± 0.00^{b}

Values are means and standard errors of 3 replicates with 10 explants per petri plate repeated 3 times within genotypes for each treatment. Means followed by the same letter in each column are not significantly different from each other (Tukey's test; $p \leq 0.05$)

Table 2: Regeneration frequencies of adventitious buds induced in 0.15mg/l TDZ from seven sweet potato genotypes in media supplemented with NAA.

Genotype	Light			Dark		
NAA (mg/l)	0	0.1	0.25	0	0.1	0.25
Enaironi	0.00 ± 0.00^{b}	20.00 ± 1.95^{d}	26.67 ± 1.36^{b}	0.00 ± 0.00^{b}	50.00 ± 2.70b^{c}	20.00 ± 2.15^{b}
Jewel	20.00 ± 1.89^{a}	60.00 ± 2.69^{a}	40.00 ± 3.42^{a}	26.67 ± 3.91^{a}	83.33 ± 2.86^{a}	40.00 ± 3.12^{a}
K. Nyerere	26.67 ± 2.76^{a}	60.00 ± 2.26^{a}	16.67 ± 1.83^{b}	20.00 ± 2.88^{a}	80.00 ± 3.29^{a}	20.00 ± 3.48^{b}
KSP36	0.00 ± 0.00^{b}	16.67 ± 2.14^{d}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	40.00 ± 3.75^{c}	26.67 ± 3.14^{b}
Kemb36	0.00 ± 0.00^{b}	33.33 ± 1.35^{c}	20.00 ± 1.42^{b}	0.00 ± 0.00^{b}	60.00 ± 3.31^{b}	40.00 ± 2.18^{a}
Mugande	0.00 ± 0.00^{b}	50.00 ± 2.17^{b}	26.67 ± 1.56^{b}	0.00 ± 0.00^{b}	50.00 ± 1.83^{bc}	0.00 ± 0.00^{c}
SPK004	0.00 ± 0.00^{b}	36.67 ± 1.35^{c}	0.00 ± 0.00^{c}	16.67 ± 1.48^{a}	50.00 ± 2.88b^{c}	0.00 ± 0.00^{c}

Values are means and standard errors of 3 replicates with 10 explants per petri plate repeated 3 times within genotypes for each treatment. Means followed by the same letter in each column are not significantly different from each other (Tukey's test; $p \leq 0.05$)

Table 3: Regeneration frequencies of adventitious buds induced in 0.25mg/l TDZ from seven sweetpotato genotypes.

observed to be genotype dependent. This could be due to the fact that different cells within the same explant can have varying concentrations of innate Plant Growth Regulators (PGRs) and additional variation in receptor affinity or cellular sensitivity to PGRs hence varying *in vitro* responses within genotypes [20]. *In vitro* regeneration is mainly regulated by the balance and the interaction between PGRs in the medium and those endogenously produced by the explants [21]. Thus, the type of PGRs in culture medium and endogenous level of the same and their interaction are important factors for regeneration of shoots and roots from explants. Generally, plant *in vitro* regeneration should be optimized for each individual genotype to achieve the best results [22].

In this study low NAA concentrations stimulated shoot formation with variable regeneration frequencies that was genotypic dependent. However, high NAA concentration levels decreased sharply the regeneration frequency due to inhibition of root formation. Response of stem explant to varying NAA concentrations in the media could be a reflection of probable differences of endogenous hormonal levels in the explant or tissue sensitivity to plant growth regulator [23]. Throughout this study, plants that were normal in appearance (without vitrification) and fertile were obtained (Figure 1f) due to the relatively short period of exposure of the explant to TDZ during the induction phase.

Conclusion

From this study six Kenyan sweetpotato genotypes were regenerated through adventitious bud regeneration protocol successfully. The result revealed that the induction and regeneration frequencies were genotype dependent for the genotypes tested. Additionally, we also demonstrated that photoperiod preference was also genotype dependent for sweetpotato regeneration.

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Competing Interests

The authors declare that they have no competing interests.

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