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THIDIAZURON IMPROVES ADVENTITIOUS BUD AND SHOOT REGENERATION IN RECALCITRANT SWEETPOTATO

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ABSTRACT

Induction of adventitious buds and shoots from intact leaves and stem internode segments of two recalcitrant Ugandan sweetpotato (*Ipomoea batatas* L.) cultivars was investigated *in vitro* on Murashige and Skoog (MS) medium, supplemented with 3 different levels (0.5, 2.0 and 4.0 µM) of Thidiazuron (TDZ). Shoots were regenerated in all TDZ concentrations in cvs. Kyebandula and Bwanjule. The inclusion of 0.25 µM α-Naphthalene acetic acid (NAA) in MS medium, containing TDZ (0.5 µM), improved shoot regeneration frequency from 12.1 to 22.6% for cv. Kyebandula stems and from 21.61 to 42.9% for cv. Bwanjule stems. However, there was about 10% reduction in adventitious bud induction frequency for both cultivars, when NAA was included in the medium. The highest frequency (66.7%) of adventitious bud induction was achieved from stem explants of cv. Kyebandula. The conversion of adventitious buds into shoots was improved when TDZ was reduced or completely removed in subsequent stages of culture. The number of explants forming shoots was significantly (P<0.001) higher when stem explants were cultured for 7 days on TDZ-supplemented MS medium before transfer to TDZ-free MS medium supplemented with NAA. Stem internode pieces from position 3 were the best (70.0%) in adventitious bud formation. However, most buds (76.2%) were not converted to shoots. The most important application of the *de novo* regeneration protocol developed in this study is in genetic transformation for improvement of sweetpotato productivity.

Key Words: Bud induction, *Ipomoea batatas*, Naphthalene acetic acid

RÉSUMÉ

L' induction des bourgeons adventices et des pousses à partir des feuilles intacts et des tiges des segments internodes de deux patates douces (*Ipomoea batatas* L.) Ugandaises recalcitrantes était étudiée en milieu *in vitro* sur le média Murashige et Skoog (MS) supplémenté avec 3 niveaux différents (0.5, 2.0 et 4.0 µM) de Thidiazuron (TDZ). Les pousses étaient régénérées dans toutes les concentrations TDZ dans les cvs. Kyebandula et Bwanjule. L'inclusion de 0.25 µM α-Naphthalène d' acide acétique (NAA) dans le média MS contenant le TDZ (0.5 µM) a amélioré la fréquence de la rénégénération des pousses de 12.1 à 22.6% pour les pousses cv. Kyebandula et de 21.61 à 42.9% pur les pousses cv. Bwanjule. Cependant, il y a eu environ 10% de réduction en termes de la fréquence d'induction des bourgeons adventices pour les deux cultivars lorsque NAA était inclu dans le média. La fréquence la plus élevée (66.7%) d'induction des bourgeons adventices était réalisée des explants des tiges de cv. Kyebandula. La conversion des bourgeons adventices en pousses était améliorée lorsque TDZ était réduit ou complètement enlevé des étapes de cultures subséquentes. Le nombre d'explants formant les pousses était significativement (P<0.001) supérieur lorsque les explants des tiges étaient cultivés pendant 7 jours sur le média MS supplémenté par TDZ avant le transfert dans le média MS sans TDZ avec pour supplément NAA. Les morceaux de tiges internodes de la position 3 étaient le meilleur (70.0%) en formation de bourgeons adventices.

Cependant, plus de bourgeons (76.2%) n'étaient pas converties en pousses. L'application la plus importante du protocol de la régénération *de novo* dévéloppée dans cette étude reside dans la transformation génétique pour l'amélioration de la productivité de la patate douce.

Mots Clés: Induction de bourgeons, *Ipomoea batatas*, acid acétique Naphthalène

INTRODUCTION

Sweetpotato (*Ipomoea batatas* (L.) Lam.), of family Convolvulaceae, is grown in the tropical and subtropical areas of the world on about 9 million hectares, with an average yield of 13.7 t ha^{-1} (FAOSTAT, 2006). Its value as food and feed is mainly due to the expanded roots, which have high content of starch, lysine, vitamin A and ascorbic acid. Sweetpotato is also an alternative source of bio-energy for production of fuel, and provides industrial material in form of starch, flour, glucose and alcohol.

The improvement of sweetpotato traits like resistance to weevils and virus diseases by conventional hybridisation has been limited because the crop is a hexaploid and has problems such as pollen sterility, cross incompatibility, poor seed germination and special physiological requirements for flowering. Genetic transformation holds potential for the improvement of sweetpotato particularly for introducing important genes that have not been hitherto identified in the sweetpotato germplasm (Kreuze *et al.,* 2008).

The successful production of transgenic sweetpotato depends on an efficient regeneration protocol (Santa-Maria *et al.,* 2009). Various explants have been used in order to improve regeneration of transgenic sweetpotato, including whole leaves (Luo *et al.,* 2006), stem internode segments (Song *et al.,* 2004), embryogenic suspension cultures (Yu *et al.,* 2007), petiole protoplasts (Dhir *et al.,* 1998), shoot meristems (Chen *et al.,* 2006), storage root discs (Newell *et al.,* 1995), leaf discs (Moran *et al.,* 1998) and axiliary buds (Oggema *et al.,* 2007). The protocols of regeneration that have shown potential for application in genetic transformation of sweetpotato include somatic embryogenesis (Song *et al.,* 2004; Kreuze *et al.,* 2008) and organogenesis (Gosukonda *et al.,* 1995a, Luo *et al.,* 2006; Santa-Maria *et al.,* 2009). An important advantage with somatic embryogenesis is that it improves the efficiency of selection of transformed plants on media (Song *et al.,* 2004). However, somatic embryogenesis remains a difficult process to control for the regeneration of sweetpotato *in vitro* (Song *et al.,* 2004; Yu *et al.,* 2007).

Most somatic embryogenesis protocols are cultivar dependent, difficult to reproduce, have low regeneration frequencies, and require long periods of culture and frequent media changes (Yu *et al.,* 2007; Kreuze *et al.,* 2008; Anwar *et al.,* 2010). Although organogenesis has shown to be easier to manipulate *in vitro,* most of the regenerated plants after transformation are escapes surviving on selection medium (Luo *et al.,* 2006). Thus, there is need for more efforts to explore adventitious shoot regeneration for the application of genetic transformation of *I. batatas*.

Thidiazuron (TDZ) has been used as a plant growth regulator to induce organogenesis, including adventitious regeneration of transformed plants in many plant species that were thought to be recalcitrant to regeneration (Cuenca *et al.,* 2000; Corredoira *et al.,* 2008; Sriskandaraj and Lundquist, 2009). In recent years, interest has grown for the application of TDZ in both adventitious and somatic embryogenesis regeneration of plants. Originally, TDZ, a synthetic phenylurea-type plant growth regulator, was considered as a cytokinin inducing responses similar to those caused by natural cytokinins (Guo *et al.,* 2011). However, TDZ is able to induce both cytokinin and auxin morphogenic responses (Jones *et al.,* 2007). It can affect meristem induction, cause shoot development from pre-formed meristems and induce adventitious bud and shoot formation in different plant species including recalcitrant woody plants (Cuenca *et al.,* 2000). Pretreatment with TDZ can predispose a tissue to accept other inductive stimuli (Gou *et al.,* 2011). Alternately, exposure to TDZ can commit a tissue to regenerative route that is expressed even after the inductive stimulus is removed.

This study was conducted to investigate the effect of TDZ on morphogenesis of *I. batatas* with the aim of inducing adventitious shoots. The ultimate goal of this study is to use the regeneration protocol in genetic transformation of sweetpotato to improve its traits, particularly resistance to weevils, for which the genes have already been identified (Ekobu *et al.,* 2010).

MATERIALS AND METHODS

Preparation of plant material and culture conditions. Two popular Ugandan sweetpotato cultivars, namely Kyebandula and Bwanjule, were used in this study. Vigorously growing vines from screenhouse plants were used to provide cuttings for establishment of *in vitro* stock cultures. The cuttings were sterilised according to the protocol of Song *et al.* (2004), except for increased immersion time in sterilising solution to from 15 to 20 minutes since a low concentration (2.0%) of NaOCl was used. The sterile cuttings were then inserted into sweetpotato propagation (SP) medium in magenta boxes.

The medium was composed of MS (Murashige and Skoog, 1962) salts premix (4.3 g l^{-1}), sucrose (30 g l^{-1}), myo-inositol (0.1 g l^{-1}), Indole-3 acetic acid (IAA) (1.0 µM), 5 ml l^{-1} sweetpotato vitamin stock comprised of 40 g l^{-1} Ascorbic acid, 20 g l^{-1} L-arginine, 4 g l^{-1} putrescine HCl, 5.8 µM gibberellic acid (GA3) and 0.4 g l^{-1} calcium pantothenate (Kreuze *et al.,* 2008). All reagents used were supplied by Sigma-Aldrich Chemie GMBH, Eschenstrasse, Taufkirchen. The medium was adjusted to pH 5.8 before adding 3 g L^{-1} phytagel, followed by autoclaving at 121 °C for 15 minutes under 15 kPa.

Adventitious bud induction and regeneration on medium with various concentrations of TDZ. The first experiment for the current study was conducted to assess the effect of various concentrations of TDZ on induction of adventitious buds and and the conversion of adventitious buds to shoots in *I. batatas.* Bud induction medium was composed of MS basal salts, sucrose (30 g l^{-1}), myo-inositol (0.1 g l^{-1}) and sweetpotato vitamin stock (1 ml l^{-1}). Various concentrations of TDZ (0.5, 2.0 and 4.0 µM) were added to the medium after autoclaving.

Intact leaves with petiole (1.0 - 1.5 cm long) and stem internode segments (0.6 - 1.0 cm) were cut from 4-week-old *in vitro* cultures growing in SP medium in magenta boxes. The stem internode segments and leaves (with adaxial side facing up) were cultured on 25 ml of a semi-solid bud induction medium in plastic petri dishes. The base of the petiole was partially embedded into the bud induction medium, while the stem internode pieces placed horizontally on the medium were partially pressed into the medium.

The petri-dishes containing the cultures were placed in dark for 4 weeks at 25 °C to induce adventitious buds before transfer to 16 hours photoperiod under the same temperature for shoot regeneration as reported by Cuenca *et al.* (2000). The cultures were transferred onto fresh medium of the same composition as bud induction medium every 4 weeks. This duration was sufficient to ensure that the media components were not severely degraded.

Adventitious bud induction and regeneration on medium with TDZ and auxin. Due to low frequency of conversion of adventitious buds into shoots, a second experiment was conducted to determine the effect of the auxin, α-Naphthalene acetic acid (NAA), on conversion of adventitious buds into shoots. In this experiment, each of the three TDZ concentrations investigated earlier was added to MS medium in combination with the NAA (0.25 µM). The inclusion of NAA in the bud induction media was based on previous reports of regeneration from recalcitrant woody plant species (Corredoira *et al.,* 2008; Sriskandaraj and Lundquist, 2009).

Adventitious bud formation and regeneration from internodes of different ages. A third experiment was carried out in order to determine whether bud regeneration frequency could be affected by internode position (age) on the mother plant. Cv. Kyebandula which exhibited high regeneration frequency was used. Five internode positions were distinguished, with node 1 being the apical-most node with unfolded leaves and node 5 the lowest from the shoot apex. The stem internodes were cut off from the mother plant as described above. These were grouped according to their position on the mother plant

before culturing in petri dishes labeled according to that position. The bud induction medium was supplied with 4.0 µM TDZ combined with 0.25 µM NAA. The petri-dishes containing the cultures were sealed and cultured for four weeks in dark, followed by transfer to 16 hours photoperiod as described above.

Duration of explants on TDZ medium, adventitious bud formation and plant regeneration. In this experiment, only stem internodes from positions 2, 3 and 4 of cv. Kyebandula were explanted. The internode pieces were explanted on medium containing only 4.0 µM TDZ as the only plant growth regulator (PGR). In order to assess the effect of duration of explants on medium containing TDZ, half of the explants were placed on the medium with TDZ for only 3 days; while the remaining half was allowed to stay on this medium for 7 days. After the designated period on TDZ-based medium, each group of explants was further divided into two equal groups and one group was transferred to medium containing with 0.25 µM NAA while the remaining one group of the explants was transferred to PGR-free medium.

Experimental design and statistical analysis. All experiments were laid out in a completely randomised design. Three petri-dishes, each containing 10 explants, were used in each experiment. This gave a total of 30 explants for each experiment. These experiments were repeated three times. After 4 weeks in culture, data on total number of explants with adventitious shoot buds and number of buds per explants were recorded. The data on number of explants with adventitious shoots (with well developed leaves and rooted), number of adventitious shoots per explants, number of explants with roots and number of roots per explants were collected after 12 weeks.

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. The frequency data were transformed using the arcsine square root before analysis to stabilise the variance. Statistical analyses were done using analysis of variance (ANOVA) and means were compared using the least significant difference (LSD) test at the $P<0.05$ level.

RESULTS

Adventitious bud induction and regeneration on medium with various concentrations of TDZ. After three days of placement on bud induction medium, explants started showing signs of expansion. The swelling was more pronounced at the cut ends of stem explants and at the cut base of the petioles of leaf explants (Fig. 1). Most explants formed non-regenerative callus at the cut ends within 2 weeks of placement on the bud medium. This phenomenon was evident in all concentrations of TDZ (data not presented).

All of the TDZ treatments induced multiple adventitious buds from the explants within the same 2 weeks (Table 1). In general, the number of stem explants that induced adventitious buds was higher than that for leaf explants. The highest number of buds was formed on 4.0 µM TDZ for both cultivars. The mean number of stem segments that formed buds on 4.0 µM TDZ was 67% for cv. Kyebandula and 59% for cv. Bwanjule. The lowest number of explants that induced adventitious buds was recorded for 0.5 µM TDZ. The number of buds per explants increased with TDZ concentration (Table 1). The number of explants producing adventitious buds and the number of buds per explant varied significantly ($P<0.001$) with both explant type and concentration of TDZ. Type of cultivar only showed a significant ($P<0.01$) effect on the number of buds formed per explants.

Shoots were regenerated in all TDZ concentrations, from both types of explants and both cultivars; although overall the buds developing into shoots were low. The highest mean number of explants forming shoots was 17.5% on 2.0 µM TDZ for stem explants of cv. Kyebandula and 21.6% on 0.5 µM TDZ for cv. Bwanjule. This high shoot regeneration was achieved for stem explants.

None of the investigated factors showed significant effect on number of explants regenerating shoots. Type of explant was the only factor that had a significant ($P<0.001$) effect on number of shoots per explant.

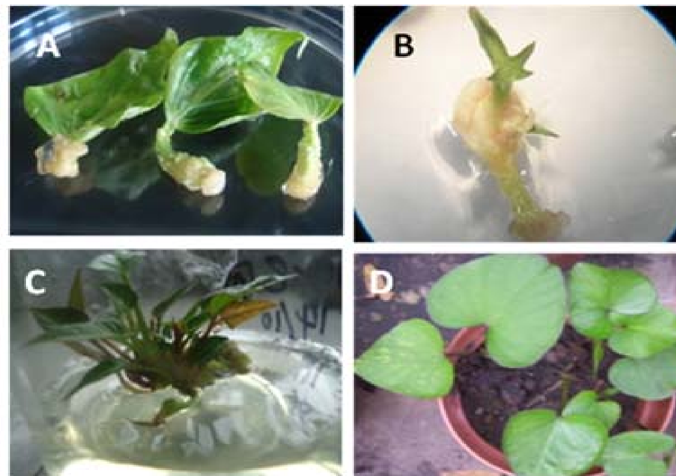

Figure 1. Adventitious bud induction, adventitious shoot regeneration and plant development in *Ipomoea batatas*: (A) Adventitious bud protrusion along the length of petiole of leaf explants after 2 weeks on bud induction MS media with 4.0 µM TDZ followed by another week on MS medium containing 0.25 µM NAA, (B) Elongation of adventitious buds leading to development of shoots on stem internode explants, (C) Development of multiple adventitious shoots on stem internode explants, (D) Growth of plants in soil 4 weeks after acclimatisation in the greenhouse.

Adventitious bud induction and regeneration on medium with TDZ and auxin. Culturing explants on medium containing both TDZ and NAA reduced the frequency of explants regenerating adventitious buds and number of buds per explants. However, the same medium increased the mean number of explants forming shoots and the number of shoots per explant, compared with cultures where TDZ was supplied alone. In addition, like for treatments with TDZ alone, 4.0 µM TDZ was the best concentration at inducing explants to differentiate multiple buds. The bud induction frequency for stem explants was 66.7 and 58.9%, for cv Kyebandula and cv Bwanjule, respectively, when TDZ (4.0 µM) was supplemented alone. However, the bud induction frequency dropped to 58.8 and 48.9%, for cv Kyebandula and cv Bwanjule, respectively, when TDZ (4.0 µM) was supplemented together with NAA.

On the other hand, the presence of NAA improved the frequency of conversion of adventitious buds into shoots, especially in the presence of low concentration (0.5 µM) of TDZ. Shoot regeneration frequency improved from 12.1 to 22.6% for cv Kyebandula, when NAA was added to the TDZ-based medium. The increase in shoot regeneration frequency for cv Bwanjule under the same conditions was from 21.6 to 42.9%. Stem explants consistently performed better than leaf explants for all variables investigated (Table 2). Type of explants was significant ($P<0.05$) in affecting the mean number of explants regenerating shoots and the number of shoots per explant. However, these two variables were not affected by type of cultivar and concentration of TDZ in NAA-containing medium. All the three factors, TDZ concentration, type of plant organ and cultivar, had a significant ($P<0.05$) effect on the frequency of explants forming buds and the mean number of adventitious buds per explant (Table 2).

Adventitious bud formation and regeneration from internodes of different ages. Adventitious buds and shoots were induced from all five internodal positions investigated (Figs. 2 and 3). Internode position 3 gave the best results; while the oldest internode (position 5) was the least responsive followed by the youngest (position 1) internode. Internode position was significant ($P< 0.05$) in affecting the mean number of explants differentiating buds, the number of buds per explant, the number of explants regenerating shoots and the number of shoots per explant.

TABLE 1. Effect of TDZ concentration (μM), types of explants of cultivar on bud induction and shoot regeneration frequency

PGR (μM)	Cultivar	Frequency (% explants regenerating buds)		Buds per explant (No.)		Frequency (% explants regenerating shoots)		Shoots per explant (No.)	
		Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf
TDZ (0.5)	Kyebendula	44.4 ± 4.8b	22.2 ± 7.8b	4.7 ± 0.9b	2.3 ± 1.2b	12.1 ± 12.12	16.7 ± 16.67	0.7 ± 0.7	0.3 ± 0.3
TDZ (2.0)	Kyebendula	51.1 ± 2.9b	20.0 ± 5.1b	7.3 ± 0.33a	5.3 ± 1.2a	17.47 ± 2.34	14.07 ± 7.07	1.2 ± 0.6	0.3 ± 0.3
TDZ (4.0)	Kyebendula	66.7 ± 8.4a	41.1 ± 2.2a	9.3 ± 0.9a	6.0 ± 1.5a	8.22 ± 2.79	3.03 ± 3.03	1.7 ± 0.7	0.3 ± 0.3
TDZ (0.5)	Bwanjule	38.9 ± 6.2b	18.9 ± 2.9b	4.33 ± 0.7b	1.7 ± 0.9b	21.61 ± 14.2	0.00 ± 0.00	1.0 ± 0.6	0.3 ± 0.3
TDZ (2.0)	Bwanjule	47.8 ± 1.1b	20.0 ± 0.0b	4.3 ± 0.7a	1.7 ± 0.9a	9.21 ± 2.06	5.56 ± 5.56	1.0 ± 0.6	0.3 ± 0.3
TDZ (4.0)	Bwanjule	58.9 ± 4.4a	37.8 ± 5.6a	6.3 ± 1.2a	5.0 ± 0.6a	3.98 ± 2.03	2.56 ± 2.56	0.7 ± 0.3	0.3 ± 0.3

Data represent means ± standard errors for three replicates (30 explants each replicate)
Mean separation within columns and treatments by Least Significant Difference (LSD) at $P<0.05$, whereby means associated with different letters indicate significant differences
Mean separation was only done for plant growth regulator (PGR) as this is the only treatment with more than two levels

TABLE 2. Effect of TDZ concentration (μM) in the presence of NAA (0.25 μM) on bud induction and shoot regeneration frequency from two types of explants of two cultivars

PGR (μM)	Cultivar	Frequency (% explants regenerating buds)		Buds per explant (No.)		Frequency (% explants regenerating shoots)		Shoots per explant (No.)	
		Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf
TDZ (0.5)	Kyebendula	40.0 ± 9.62b	23.3 ± 3.85b	4.3 ± 0.3b	2.0 ± 0.6b	22.6 ± 8.5	34.0 ± 3.3	2.0 ± 0.0	1.6 ± 0.3
TDZ (2.0)	Kyebendula	41.1 ± 4.84ab	23.3 ± 3.85ab	8.0 ± 1.0b	5.7 ± 0.7b	28.3 ± 20.8	38.0 ± 14.3	2.7 ± 0.9	2.3 ± 0.3
TDZ (4.0)	Kyebendula	58.9 ± 7.29a	42.2 ± 2.22a	8.0 ± 1.5a	4.3 ± 1.5a	21.2 ± 2.7	11.1 ± 5.6	2.0 ± 0.0	1.2 ± 0.6
TDZ (0.5)	Bwanjule	31.1 ± 4.01b	21.1 ± 2.22b	3.3 ± 0.7b	2.0 ± 0.6b	42.9 ± 14.3	6.7 ± 6.7	1.8 ± 0.2	0.3 ± 0.3
TDZ (2.0)	Bwanjule	40.0 ± 1.92ab	15.6 ± 4.01ab	2.3 ± 0.7b	2.0 ± 0.6b	17.5 ± 9.5	38.1 ± 31.2	1.7 ± 0.3	2.0 ± 1.5
TDZ (4.0)	Bwanjule	48.9 ± 11.60a	27.8 ± 5.88a	4.7 ± 1.5a	4.3 ± 1.9a	8.5 ± 4.3	26.1 ± 8.4	1.5 ± 0.9	1.5 ± 0.3

Data represent means ± standard errors for three replicates (30 explants each replicate)
Mean separation within columns and treatments by Least Significant Difference (LSD) at $P<0.05$, whereby means associated with different letters indicate significant differences
Mean separation was only done for Plant Growth Regulator (PGR) as this is the only treatment with more than two levels

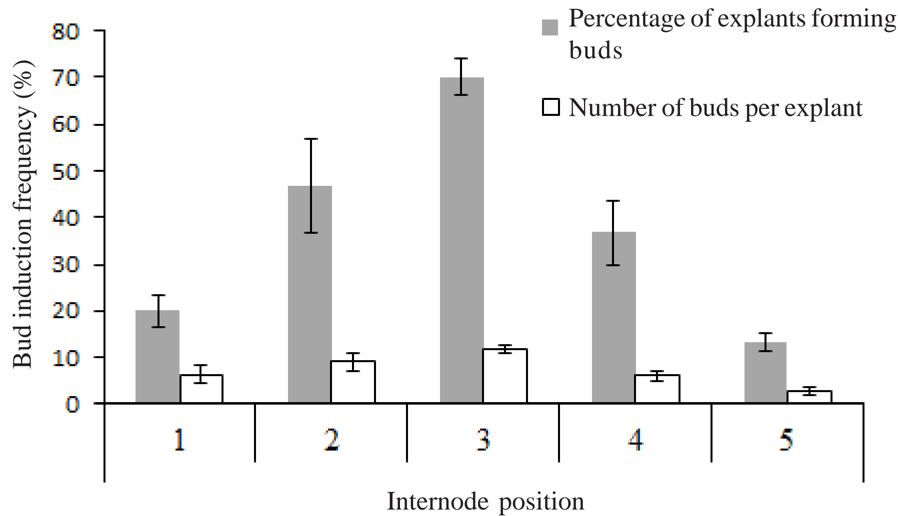

Figure 2. Effect of internode position on number of explants forming adventitious buds and number of adventitious buds per explant. Five internode positions were distinguished along the shoot with internode 1 being at the shoot apex and internode 5 being the lowest from the shoot apex. Data represent means from three replicates (30 explants in each). Vertical lines indicate standard errors of the means. Means with different letters are significantly different at P<0.05 (LSD test).

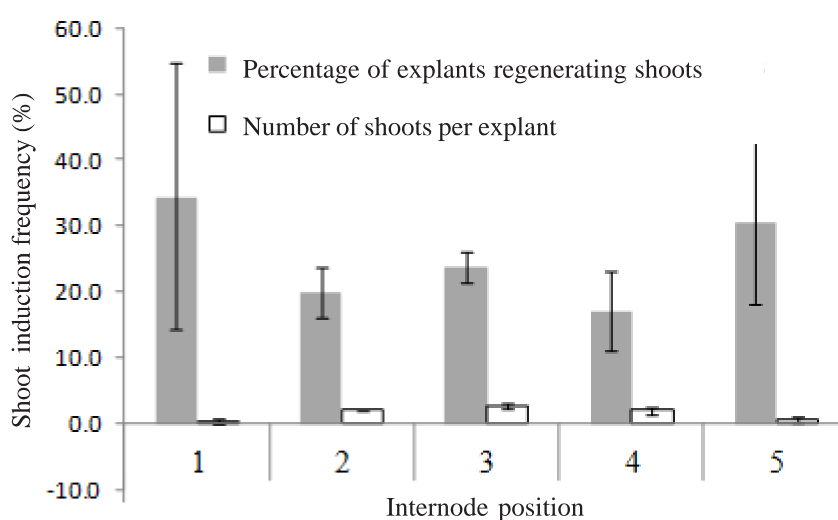

Figure 3. Effect of internode position on number of explants forming adventitious shoots and number of adventitious shoots per explant. Five internode positions were distinguished along the shoot with internode 1 being at the shoot apex and internode 5 being the lowest from the shoot apex. Data represent means from three replicates (30 explants in each). Vertical lines indicate standard errors of the means. Means with different letters are significantly different at P<0.05 (LSD test).

Duration of explants on TDZ medium, adventitious bud formation and plant regeneration. The best results were obtained when stem internode segments were cultured on bud induction medium (4.0 µM TDZ) for 7 days followed by cultures on medium containing 0.25 µM NAA. The 4.0 µM concentration of TDZ was best for adventitious bud induction according to initial experiments described above. The removal of TDZ from media without supplying NAA resulted in reduction of both bud and shoot regeneration. The highest frequency (61.1%) of explants differentiating adventitious buds was achieved when duration on TDZ was increased

from 3 to 7 days before transfer to NAA medium (Table 3). The number of explants-forming shoots was also significantly (P<0.001) higher for this treatment. However, the number of shoots per explant was not significantly affected by duration on TDZ or subsequent placement on either NAA or PGR-free medium. These results show that the frequency of explants inducing adventitious buds and the number of adventitious buds per explants was higher than the best of the experiments on effect of supplementing 0.25 µM NAA together with TDZ in medium (Tables 2 and 3).

DISCUSSION

Adventitious bud and shoot regeneration of sweetpotato (*Ipomoea batatas)* was achieved from two recalcitrant cultivars, Bwanjule and Kyebandula within 12 weeks (Table 1). However, the number of plants regenerated using the reported protocol were highly dependent on the number of explants investigated since the number of shoots regenerated per explant was considerably low. Nevertheless, the results achieved in this study are very important for the breeding of *I. batatas*, particularly African cultivars, which have been reported to be difficult to regenerate through both somatic embryogenesis and organogenesis; and are still considered recalcitrant to *in vitro* regeneration (Luo *et al.,* 2006). The plants regenerated in the present study do not originate from pre-formed buds but *de novo* buds induced by TDZ. It is important that direct regeneration without an intervening callus stage should be of single cell origin, avoiding possibility of chimeras after genetic transformation (Gong *et al.,* 2005).

Recently, there has been increased application of TDZ in somatic embryogenesis and adventitious regeneration of recalcitrant plants, particularly woody species (Cuenca *et al*., 2000; Corredoira *et al.,* 2008; Sriskandarajah and Lundquist, 2009). However, the use of TDZ-based protocols for the regeneration of *I. batatas* has only been reported by few workers (Gosukonda *et al.,* 1995a; Gosukonda *et al.,* 1995b; Kumar *et al.,* 2007). When explants were cultured on TDZ-supplemented medium, multiple adventitious buds were induced along the length of explants. Some of the buds successfully elongated into shoots that developed into plants (Fig. 1). These observations were common for all TDZ concentrations (Table 1). Kumar *et al.* (2007) reported regeneration through adventitious shoots on media containng cytokinins TDZ, benyladenine or 2-isopentenyladenine singly or TDZ combined with Indole acetic acid (IAA).

Although preliminary studies with 45.0 µM TDZ led to regeneration of sweetpotato from cultivar Kyebandula, most of the explants died after turning brown within 4 weeks (data not presented). In spite of the observation that all trials with TDZ led to regenerants, higher doses of TDZ inhibited further growth and development of the regenerants (Table 1).

It was recently shown that shoot induction is achieved at range 1.0 nM to 10.0 µM TDZ (Thomas and Phillip, 2005). These workers recommended that less than 5.0 mM was optimal for organogenesis. When Gosukonda *et al.* (1995a) evaluated various TDZ concentrations

TABLE 3. Effect of duration on medium containing TDZ (4.0 µM) and transfer to NAA (0.25 µM) on bud induction and shoot regeneration frequency from stem explants

First media (duration)	Second media	Frequency (% explants forming buds)	Buds per explant (No.)	Frequency (% explants forming shoots)	Shoots per explant (No.)
TDZ (3 days)	NAA	40.0 ± 12.0ba	6.0 ± 1.0b	18.2 ± 6.6c	1.0 ± 0.6
TDZ (3 days)	PGR-Free	3.3 ± 3.3cb	1.3 ± 1.3c	0.0 ± 0.0c	0.7 ± 0.7
TDZ (7 days)	NAA	61.1 ± 4.0a	13.0 ± 1.2a	55.8 ± 7.1b	3.0 ± 0.6
TDZ (7 days)	PGR-Free	20.0 ± 3.3bc	3.7 ± 0.3ab	13.1 ± 7.2c	1.0 ± 0.6

Data represent means ± standard errors for three replicates (30 explants each replicate)
Mean separation within treatments by Least Significant Difference (LSD), P<0.05, whereby means associated with different letters indicate significant differences

(0.0 to 0.4 mg L^{-1}) for the regeneration of sweetpotato, they found that 1.0 µM (0.2 mg L^{-1}) was optimal for regeneration of adventitious shoots from two of six cultivars grown in Americas. Later, Kumar *et al.* (2007) reported that 5.0 µM (1.0 mg L^{-1}) TDZ was optimal for adventitious shoot regeneration from leaf explants of 'Tainong (T) 57', 'T 64' and 'T 66' sweetpotato cultivars from Japan.In this study, reduced concentrations of TDZ generally led to improved shoot regeneration (Table 1). The same trend was observed for number of shoots per explants for cultivar Kyebandula. Although the successful plant regeneration in this study was due to the use of TDZ, there was low frequency of conversion of adventitious buds into shoots in the presence of this plant growth regulator. Higher (2.5 and 5 µM) TDZ concentrations inhibited shoot elongation and stimulated callus production in *Phaseolus vulgaris* L. and *Vicia faba* L. (Mohamed *et al.,* 1992). Several authors reported that TDZ-induced buds do not readily elongate into shoots, a phenomenon that could be caused by high concentration of TDZ in the medium or prolonged persistence of TDZ in the explants tissues (Cuenca *et al.,* 2000; Lyrra *et al.,* 2006; Kumar *et al*., 2007; Corredoira *et al*., 2008). TDZ may be inhibiting shoot elongation due to its cytokinin activity leading to increased level of endogeneous cytokinin which inhibits effect of cytokinin oxidase and endogenous auxin (IAA) (Debnath, 2005).

When the auxin NAA (0.25 µM) was included in the current study, an increase in bothmean number of explants forming shoots and the number of shoots per explants was recorded (Table 2). Similarly, auxins like IAA and NAA, have been shown to improve adventitious regeneration and shoot organogenesis in other TDZ-based protocols (Corredoira *et al.,* 2008; Sriskandarajah and Lundquist, 2009). Although the inclusion of the auxin NAA (0.25 µM) clearly led to an increase in the number of explants forming shoots and shoots per explants in this study, the number of explants inducing buds and the number of buds per explants was affected (Table 2). This challenge was overcome by initially placing explants on medium with TDZ alone, followed by transfer to medium with NAA only (Table 3).

Although both cultivars investigated were able to regenerate plants, cultivar Kyebandula responded better than Bwanjule on all experiments. The different cultivar responses could be due to endogeneous differences in auxin and cytokinin content in the cultivars. It was also observed in the current study that stem explants were significantly better than leaf explants in both adventitious bud induction and shoot regeneration (Table 1). Gosukonda *et al.* (1995a) found leaf lamina of *I. batatas* to be the least responsive explants when compared to petioles. These workers also found TDZ better than both Zeatin riboside and Kinetin in inducing adventitious regeneration.

Although adventitious buds and shoots were induced in all internodal, internode position 3 gave the best results; while the oldest (position 5) and the youngest (position 1) gave the lowest mean numbers of buds and shoots. In general, lower morphogenic capacity (adventitious shoots) is expected as the age of the donor plant increases. When experimenting with *I. batatas* leaf explants, other workers found that only second and third leaves were the best (Dessai *et al.,* 1995). Stem internode pieces from two *Fagus* species were also shown to have highest regenerative response from internodes proximal to the apical meristem, while those distal to the apex were the least productive (Cuenca *et al.,* 2000). Differences in regenerative abilities of explants of varying ages may be a result of differences in internal auxin, cytokinin and/or abscisic acid levels.

CONCLUSION

A reliable adventitious regeneration protocol has been established for *I. batatas* cultivars, which have not been regenerated before. This protocol has potential to be extended to the regeneration of other economically important *I. batatas* cultivars. The ultimate goal is to apply this protocol in genetic transformation for improvement of *I. batatas* traits, especially for resistance to weevils. Overall, the results presented in this paper confirm that most *I. batatas* cultivars that were thought to be recalcitrant can be regenerated following optimisation of media composition.

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