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In vitro microtuberisation of two improved Ugandan *Solanum* potato varieties

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Abstract

The performance of explants derived from three and six week-old green house-grown potato (Victoria and Kisoro) mother plants was evaluated. The mother plants were fertilized with different combinations of nitrogen, phosphorus, and potassium fertilizers. Explants were cultured on modified Murashige and Skoog's(MS) basal salts, supplemented with appropriate vitamins and growth regulators, for shoot and microtuber induction. Microtubers were induced directly from nodal cuttings inoculated on tuberisation medium and from defoliated *in vitro* shoots previously grown on shoot propagation medium, before transfer onto the tuberisation medium. *In vitro* shoot length and node number were significantly ($P<0.05$) increased by sole N application and retarded by sole P, or K application. Application of N along with P or K showed intermediate effects, but improved shoot growth. Single N application was inimical to microtuber formation, whereas P and K promoted early tuberisation, characterised with significantly ($P<0.05$) large diameters and fresh weights. Victoria produced bigger and fewer microtubers than Kisoro. Increased number of microtubers occurred due to combined application of N,P and K. Microtubers derived from defoliated *in vitro* shoots showed no significant ($P>0.05$) difference in size due to mother plant pre-treatment, unlike the number of microtubers per explant. The six week-old nodal cuttings barely regenerated (5-15%) on the tuber induction medium, and had 30-70% regeneration on shoot induction medium. Better regeneration (60-70%) occurred in the N fertilized mother plants than with P and K (30-50%). Microtubers derived from defoliated *in vitro* shoots, irrespective of genotype and pre-treatment produced very few, tiny microtubers and others did not tuberize. Attempts to chemically break dormancy using gibberellic acid gave eratic results. No significant variation was observed between the conventional mother clones and the *in vitro* cultures and microtubers.

Keywords: *Solanum tuberosum*, nitrogen, phosphorus, potassium, microtubers, dormancy, gibberellic acid.

Introduction

In Uganda, the potato (*Solanum tuberosum* L.) is now considered one of the important food and high-value commercial crops (Sikka et al., 1994). Although its cultivation was originally confined to the highland areas, the crop is now grown in most parts of the mid and low altitudes, because promising genotypes suitable to the lowlands and tolerant to bacterial wilt (*Pseudomonas solanacearum* E.) have been screened (Sikka and Kanzikwera 1993; Sikka et al., 1994; Bhagsari et al., 1995). Despite the integrated use of conventional, rapid multiplication and true potato seed techniques for seed multiplication, demand for ware potatoes continues to outstrip production mainly due to the inadequate amount of healthy seed of proven varieties (Sikka et al., 1994). It is for this reason that work was initated to explore the use of micropropagation techniques to supplement existing multiplication methods in order to obtain increased planting material. Micropropagation has been widely used for massive multiplication of the potato (Wang and Hu 1982; Tovar et al., 1985; Dodds, 1984, 1989; Wang et al., 1992). The most popular propagation materials used are *in vitro* microtubers and shoot cultures derived from meristems and axilliary buds.

Since several factors affect *in vitro* growth and morphogenesis (Avilla et al., 1973; George and Sherrington, 1984; Seabrook et al., 1993), it is a prerequisite that conditions established should favour rapid multiplication, and methods used do not result in gross variation of the propagules from the mother plants (Wenzel, 1994). Although the medium composition phyisical enviroment affects micropropagation rate, the condition of the parent - of- origin of explant it also important. The nutrition status the parent - of - origin with regard to nitrogen, phosphorus and potassium influences *in vitro* performance (George and Sherington, 1984).

Despite the promising future for large scale production of microtubers, difficulties in dormancy break have been reported (Tovar et al., 1995), hence the need to investigate appropriate methods for dormancy break (Tovar et al., 1985; Ahloowalia, 1994; Wenzel, 1994).

Therefore, the objective of this work was to determine the effects of nitrogen, potassium and phosphorus fertilizer application to potato mother plants on the *in vitro* growth

and morphogenesis of shoots and microtubers. Investigations were also done on microtuber dormancy break and comparision of the *in vitro* propagules with conventional tubers for the purpose of monitoring clonal fidelity.

Materials and methods

The study was conducted at Makerere University Agricultural Research Institute, Kabanyolo. Pre-sprouted tubers (>3cm sprout length) of two improved potato varieties, Kisoro and Victoria, were planted in the screen house in 20 x 20 cm pots containing 3 kg of heat sterilized soil. Mother plants received 69 kg N ha^{-1} as Urea,8 kg P ha^{-1} as single super phosphate, and 40.8 kg K ha^{-1} as muriate of potash. Nitrogen and potassium were applied in splits, at planting and two weeks after planting, whereas phosphorous was applied once as a pre-plant treatment. There were eight treatments of N,P and K including one control as follows: 0, 0, 0; 69, 0, 0; 0, 8, 0; 0, 0, 40.8; 69, 0, 40.8; 69, 8, 0 and 69, 8, 40.8.

Plants were regularly watered and sprayed with as a dilute mixture of Dithane M-45 and Benlate (ratio 1:1) to control late and early blights. At weeks three and six after emergence, stems were excised and nodal cuttings bearing axillary bud at the leaf axil were isolated. The buds were selected between the third node below the terminal bud and the third last node above the stem base in order to eliminate differences in age due to explant position along the stem.

Nodal cuttings were surface-sterilized in 96% ethanol for 20 seconds, followed by 15 minutes in a mixture of (v/v) bleach solution of laundry grade Sodium hypochlorite (Reckitt and Colman Industries, Kenya), and 2-3 drops of Tween 20 at 0.2% (v/v). The segments were then rinsed three times in distilled, deionized and sterile water. Disinfected explants were trimmed to size (1-2 cm) and then aseptically transferred in a laminar flow chamber onto growth medium contained in glass baby jars (SIGMA BRAND), with one segment per bottle. Microtubers were induced directly from green house grown nodal cuttings inoculated on tuberisation medium and from *in vitro* defoliated shoots previously grown on shoot propagation medium. The medium for tuberisation contained Murashige and Skoog basal salts (MS) (1962), supplemented with 30 g l^{-1} sucrose, 1 mg l^{-1} benzyl aminopurine (BAP), 6 g l^{-1} agar, 0.25 mg l^{-1} gibberellic acid (GA_3), whereas the medium for microtuberisation contained 8% sucrose, 5 mg l^{-1} BAP, and 8 g l^{-1} agar. The media were adjusted to pH 5.8 using 0.1M sodium hydroxide solution prior to autoclaving. Approximately 15 mls of the medium were dispensed into the baby jars and sterilized at 121° C for 15 minutes at a pressure of 1.05 kg cm^{-2}. Cultures were incubated at a temperature of 25-30°C, and 16 hour photoperiod under high light irradiance (1773 $\pm$ 42 lux) for shoot growth and low light irradiance (< 887$\pm$ 42 lux) for microtuberisation. Each treatment was replicated five times, and a replicate consisted of three culture vessels. The experiment was repeated twice in a split-plot design, with varieties constituting the main plots and fertilizer treatments the sub-plots.

Data were taken on time to microtuber initiation (days), number of microtubers per explant, and microtuber diameter (mm). Percentage regeneration of explants was assessed as the number of regenerants divided by total number initiated, multiplied by 100. Analysis of variance (ANOVA) was done to separate the effect of mother plant pre-treatment on the *in vitro* microtuberisation, using two-factor split plot design of M- Statc package (Russel, 1986). Means were separated using Fisher's Protected Least Significance Difference (LSD) test at p= 0.05 (Steel and Torrie, 1980).

Dormancy period. *In vitro* microtubers (in sterile 9-cm plates sealed with parafilm) were stored in the dark at 25-30° C. Storage started immediately after the harvested microtubers of three common cultivars (Victoria, Kisoro and Sangema) received four treatment combinations derived from two levels of gibberellic acid concentrations (5 mg l^{-1} and 10 mg l^{-1}), and exposed for 15 minutes and 30 minutes. The control experiment had no gibberellic acid applied. The duration of dormancy was recorded, and defined as the period between the harvest of microtubers until 50% of them had produced a sprout of at least 2 mm (Ranalli, 1994).

Each treatment had thirty microtubers per petri dish and was replicated three times. During incubation, the number of sprouted microtubers in every batch were recorded at 30, 40 and 60 days after treatment with GA_3 and expressed as percentages of the total number of microtubers incubated per petri dish.

Analysis of variance (ANOVA) was done to determine the effect of GA_3 and time of exposure on dormancy break, using the M-Statc package (Russel, 1986). Means were separated using LSD at p = 0.05.

Evaluation of cultures for morphological characteristics. Shoot cultures were monitored starting from the test tube stage to the screen-house stage of mini-tuber production. Changes in microtuber characteristics could not easily be detected due to the light green colour they possessed, their analyses was therefore done at screen-house stage. One hundred plantlets from each of three varieties Kisoro, Sangema and Victoria were evaluated for: leaf size, leaf shape, leaf colour, flower colour, variegation, dwarfness, chlorophyll deficiency and anthocyanin deficiency. Minitubers were analysed for tuber skin colour, dormancy and shape.

One hundred microtubers of Kisoro and Victoria varieties were planted in the screen house in sterile soil contained in black polythene tubes (20 cm x 15 cm). Sangema microtubers were not considered because of their poor *in vitro* microtuberisation which resulted in insufficient numbers of microtubers for reasonable analysis. In each case, the plants were allowed to grow to maturity and minitubers were harvested. Tubers derived from field-grown plants were utilized as controls. The occurrence of off-types was recorded as percentage of the total number of plantlets used.

Results

Shoot growth and morphogenesis. For the three-week old potato mother plants the stimulatory effect of N was expressed in both varieties as significant ($p< 0.01$) increases in shoot length, and nodes per explant. Victoria had higher scores than Kisoro (data not shown). In contrast, application of P and K singly or in combination with N significantly decreased shoot length and node number. Most plantlets were stunted and not vigorous (Table 1). P and K in combination with N showed intermediate effects on shoot growth and did not differ significantly from each other ($P>0.05$). Similar trends were observed in the explants derived from six-week old mother plants (Table 2). The shoot growth declined with advance in mother plant ontogeny and regeneration decreased by approximately 20-70%, depending on the treatment. The percentage of explant regeneration for the three elements singly was, 20% (N), 45% (K), and 70%(P). Most explants, derived from mother plants which received P or K treatments, dried-up after 2-3 weeks of culture initiation with exception of only a few.

Microtuber yield and yield components

Greenhouse nodal cuttings

Microtuber induction in the 3-week old nodal cultures were significantly ($P<0.05$) delayed by sole nitrogen application to mother plants. This was not observed in P and K. Nitrogen was associated with production of stolons which in most cases, did not tuberize or had miniature microtubers (Table 3). Potassium induced tuber initiation

Table 1. Effect of N, P and K application to three weeks old potato mother plants on *in vitro shoot growth* of two varieties

N,P,K kg ha $^{-1}$	Shoot growth	
	Average shoot length(cm)	No. of nodes /shoot
0:0:0	5.70c	7.80c
69:0:0	9.10a	12.66a
0:8:0	3.20e	5.20d
0:0:40.8	4.60d	5.15d
69:0:40.8	6.90b	8.89c
69:8:0	7.12b	8.78c
0:8:40.8	4.93d	7.59c
50:8:40.8	6.88b	10.45b
CV(%)	18.12	19.30

Data are means of 160 culture vessels; means in the same column followed by the same letters are not significantly different at $P<0.05$ according to Fishers' LSD test.

Table 2. Effect of N, P and K application to six- week old potato mother plants on *in vitro* shoot growth of two varieties

N,P,K kg ha $^{-1}$	Shoot growth	
	Average shoot length(cm)	No. of nodes /shoot
0:0:0	2.64d	2.75c
69:0:0	7.28a	6.00a
0:8:0	1.04e	1.25d
0:0:40.8	1.40e	1.75d
69:0:40.8	3.08c	2.63c
69:8:0	2.89c	2.38c
0:8:40.8	1.74e	1.75d
69:8:40.8	4.95b	4.38b
CV (%)	24.97	22.26

Data are means of 160 culture vessels; means in the same column followed by the same letters are not significantly different at $p<0.05$ according to Fisher's LSD test.

Table 3. Effect of N,P and K application to potato mother[1] plants on *in vitro* microtuberisation[2] of varieties Victoria and Kisoro

NPK kg ha	Microtuber yield and yield components			
	Time of tuberisation (days)	No. of microtubers/ explant	A verage microtuber diameter(mm)	Average microtuber weight(mg)
0:0:0	17.75b	2.17d	4.5c	68.c
69:0:0	23.80a	1.03e	2.21d	39.00d
0:8:0	14.88c	1.36e	8.36a	160.30a
0:0:40.8	15.00c	1.32e	7.98a	158.22a
69:0:40.8	16.96b	2.65c	5.72b	100.78b
69:8:0	18.23b	2.70c	5.46b	104.66b
0:69:40.8	16.75bc	3.26b	7.70a	150.05a
69:8:40.8	19.00b	5.00a	5.45b	80.32c
CV (%)	27.60	25.00	16.50	23.15

Data are means from 160 culture vessels; means in the same column followed by the same letters are not significantly different at $p<0.05$ according to Fisher,s LSD test.

[1]three-week old mother plants after emergence.

[2]microtubers derived from screen house grown nodal cuttings

slightly earlier than P, however, both elements were characterized by single microtubers per explant which significantly (P>0.05) expressed the highest diameter (K, 7.98 mm; P, 8.36 mm) and fresh weight (K, 158.22 mg; P, 160.30 mg).

Application of combined N, P and K significantly (P>0.05) increased the number of microtubers per explant, but not size. Varieties significantly (p>0.05) differed in microtuber size and fresh weight, but not in the time of tuber initiation (data not shown). Victoria produced bigger microtubers as compared to Kisoro. Results of six-week old explants cultured directly in tuberisation medium were not considered since most of them did not renegerate.

Defoliated in vitro shoots

In the three-week old defoliated *in vitro* shoots, fertilizer treatments did not induce significant (p>0.05) differences in time of tuber initiation, microtuber diameter and fresh weight, except for microtuber numbers per explant (Table 4). Nitrogen was associated with a significant (p<0.05) increase in number of microtubers per explant, but not P and K. Since the N, P and K x Variety interractions were non-significant (P>0.05), only the significant N, P and K effects have been considered.

The defoliated *in vitro* shoots derived from P or K treated six-weeks old mother plants either had 1 to 2 spindly microtubers or did not tuberize (50-70%). Microtuber size was not affected by the fertilizer application except for the treatments (0:8:0) and (0:8:40:8)in which microtuber weight was significantly low (14:61 mg and 13:31 mg), respectively (Table 5). However, varietal effects were significant, with Victoria having larger tubers than Kisoro (data not shown). The

Table 4. Effect of N, P and K application to three weeks old potato mother plants on the *in vitro* microtuberisation[1] 2 of varieties

N,P,K kg ha^{-1}	Microtuber yield and yield components			
	Time of tuberisation (days)	No. of microtubers/ explant	Average microtuber diameter(mm)	Average microtuber weight (mg)
0:0:0	18.00a	2.48e	2.75a	32.97
69:0:0	16.81a	7.57a	3.80a	34.14
0:100:0	15.81a	1.68e	3.28a	32.30
0:0:40.8	15.60a	1.63e	3.08a	33.33
69:0:40.8	15.40a	5.00c	3.98a	34.20
69:8:0	17.96a	4.45c	2.33a	34.16
0:8:40.8	17.03a	3.51d	3.28a	33.00
69:8:40.8	16.85a	6.15b	3.02a	32.45
CV (%)	9.03 ns	17.62	20.6 ns	15.1 ns

Data are means from 160 culture vessels; means in the same column followed by the same letter are not significantly different at P< 0.05 according to Fisher,s LSD test.
[1]explants used are defoliated *in vitro* shoots derived from the three- week old mother plants.

Table 5. Effect of N, P and K application to six-weeks old potato mother plants on the *in vitro* microtuberisation[1] of two varieties

N,P,K kg ha^{-1}	Microtuber yield and yield components			
	Time of tuberisation (days)	No. of microtubers/ explant	Average microtuber diameter (mm)	Average microtuber weight (mg)
0:0:0	18.50a	0.72c	1.03a	15.98a
69:0:0	17.07b	3.24a	3.38a	22.21a
0:8:0	15.31a	0.79c	1.19a	14.61b
0:0:840.8	15.20b	0.88c	1.63a	17.38a
69:0:40.8	19.06a	1.40b	1.83a	19.30a
69:8:0	18.11a	1.25b	1.79a	21.77a
0:8:40.8	15.00a	0.89c	1.80a	13.31b
69:8:40.8	16.55ab	1.75b	2.90a	19.88a
CV (%)	24.00 ns	26.6	23.00 ns	19.9

Data are means from 160 culture vessels; means in the same column followed by the letter are not nificantlydifferent at p= 0.05 according to Fisher,s LSD test.
[1]explants used are defoliated *in vitro* shoots derived from six- week old mother plants.

tiny microtubers of both varieties dried up when incubated for sprout induction, showing no yield advantage.

Discussion

The suppressing effect of nitrogen supply on tuber initiation (like in the three-week old nodal cuttings, Table 3) has also been documented by Moorby (1978) and Harris (1992). It apparently acts by affecting levels of endogenous hormones that influence tuber initiation and growth. Phosphorus is known to hasten tuber initiation (Beukema and Vander Zaag, 1990; Harris, 1992), which also is in agreement with our work.

Defoliated *in vitro* shoots had more microtubers per explant than nodal cuttings, but no differences were detected for microtuber diameter and fresh weight. The effects of N, P, and K were not significant in the defoliated *in vitro* shoots, except for the number of microtubers per explant, probably due to the trace nutrient amounts remaining in the tissues. The significant increase in number of microtubers attributed to nitrogen in defoliated *in vitro* shoots may be a secondary effect due to higher adventitious shoot number, but not a direct influence of N application.

Although N promoted mother plant longevity better than P and K, delaying excition of explants until six weeks after emergence was not advantageous for quality growth and morphogenesis of adventitious shoots and microtubers. Harris (1992) reported that as potatoes increase in ontogenetic age, there is decline in concentration of N, P and K in the haulm due to depletion by the developing tubers. This occurs more in the stems than in the roots and leaves. This depletion, in addition to decline in regenerative capacity due to senescence of mother plant, affects explant regeneration and growth.

The results of this study demonstrate that mother plant longevity, *in vitro* shoot and microtuber growth and morphogenesis can be influenced by pre-treatment of the stock plants with N, P and K, but varies with advance in plant ontogeny. Practically, rapid propagation can be achieved by stimulating explants with the right nutrient and isolating explants during the productive period of mother plant development.

Dormancy period

There was no visible sprout growth observed in all the varieties 30 days after microtuber treatment with GA_3. Sprout induction, however, occurred in microtubers of all the varieties after 40 days of incubation, but even then, with less than 50% sprouting. Sprouting was more pronounced in Victoria than in Kisoro and Sangema (Table 6). For the control batches, all Sangema microtubers were dormant 40 days after chemical treatment, whereas dormancy break was achieved in Victoria (46.7%) and in Kisoro (27.3%). Regardless of the time of exposure, microtubers which received a higher concentration of GA_3 generally showed greater percentage of dormancy break, the magnitude being genotype specific. However, exposure to GA_3 for a shorter period (15 minutes) tended to favour greater dormancy break than 30 minute-exposure. This trend in the response was not consistent within and between varieties. Over 50% of the microtubers for each variety had broken dormancy sixty days after being treated with GA3, with Sangema ranking topmost followed by Victoria and Kisoro. Most of the tiny microtubers (<100 mg) dried up in the process of inducing dormancy break.

It is apparent from Table 6 that *in vitro* induced microtubers took longer to break dormancy compared to the conventional tubers, of the corresponding varieties. In a previous study Ajonye, (1996) demonstrated that for the conventional tubers, Victoria had the shortest dormancy period (30 days), whereas Sangema and Kisoro had medium dormancy durations (40 and 50 days, respectively), by which time over 90% dormancy break was attained, without enhancing them chemically or physically. Difficulties in microtuber dormancy break have been reported by Tovar et al. (1985), Ahlowalia (1994), Seabrook (1993) and Wenzel (1994). Bryan (1989) observed erratic response in dormancy break due to various

Table 6. Mean percentage dormancy break in microtubers of Victoria, Kisoro and Sangema exposed to different concentrations of gibberellic acid and durations.

Treatments GA_3= mg/l (time= minutes; GA_3= mg/l	Victoria		Kisoro		Sangema	
	40 days	60 days	40 days	60 days	40 days	60 days
Control	46.7b	76.7a	27.3b	72.7a	0.0c	66.7c
T= 15; GA3= 5	53.8a	84.4a	28.6b	48.6c	0.0c	90.0a
T= 15; GA3= 10	57.1a	64.3b	36.4a	54.5b	0.0c	92.0a
T= 30; GA3= 5	41.7b	75.0a	40.0a	60.0b	55.6a	66.7c
T= 30; GA3= 10	50.0ab	66.7b	30.0ab	60.0b	62.3a	80.4b
CV (%)	30.0	28.3	28.5	27.7	25.4	26.2

Data are means from 45 petri dishes replicates; means in the same column followed by the same letters are not significantly different at $p< 0.05$ according to Fishers LSD test.

chemical pre- treatments (including GA_3) in conventional potato tubers. He noted that different types of genetic materials react differently to chemicals that promote sprouting. It is thought that microtuber dormancy is related to the hormonal balance within the tuber (Bryan, 1989), thus the different methods of *in vitro* tuber induction and enviromental conditions play a regulatory role in controlling *in vitro* tuber dormancy.

Culture evaluation

Of the ninety plantlets analysed, none of them deviated in morphological characteristics from the mother clones, except for two plantlets from varieties Kisoro and three of Victoria which showed purpling at leaf edges. However, after weaning in the screen house, the colours disappeared. This could be an epigenetic change rather than somaclonal variation because of the property of reversibility. According to De Klerk (1990), one of the characteristics of an epigenetic change is that the variation is reversible during the life time of a plant whereas somaclonal variation is not.

These observations show that production of microtubers and shoot cultures with this technique is feasible for rapid propagation of the improved varieties tested in this experiment. Similar experiments were done by Ahloowalia (1994) on microtubers and minitubers and, on non-irradiated shoot cultures (Sonnino, 1985) where they observed no morphological change in the characters. However, the failure to observe gross changes does not negate the possibility of genetic variations which more sensitive detection techquenics could have revealed. One of the problems encountered was that of poor emergence, growth and minituber yield by tinny microtubers (less than 2 mm in diameter).

Although Ranalli et al. (1994) observed no gross morphological variations in plants derived from microtubers, they noticed that in smaller microtubers (less than 3mm in diameter) emergence and growth were much retarded and better minituber yields were obtained from larger microtubers. Therefore, it is essential to know the genetic background of materials before selecting a particular method or chemical for breaking dormancy.

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