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Full Length Research Paper

Selection of an efficient *in vitro* micropropagation and regeneration system for potato (*Solanum tuberosum* L.) cultivar Desirée

Mahmoud M. Saker[1], Tarek A. A. Moussa[2]*, Nahed Z. Heikal[2], Amany H. A. Abo ELLil[2] and Rehab M. H. Abdel-Rahman[2]

[1]Plant Biotechnology Department, Genetic Engineering and Biotechnology Division, National Research Center, Egypt.
[2]Botany Department, Faculty of Science, Cairo University, Giza 12613, Egypt.

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Sprouts of about 40 to 80 mm length were excised, surface sterilized with 70% Clorox® and cultured on solid full-strength Murashige and Skoog (MS) medium. Shoot nodal segments (1.0 cm) from *in vitro* plantlets (2 to 4 weeks old) were multiplied through periodic subculturing on full-strength MS medium with 30 g/L sucrose, 100 ml/L myo-inositol and 0.5 ml/L silver thiosulfate. The shoots were rooted on the same medium. Microtubers were stimulated on MS medium supplemented with 80 g/L sucrose, 100 ml/L myo-inositol and 5 ml/L benzyl adenine. They generally originate on aerial etiolated shoots producing ≈ 1.0 ± 0.5 microtuber/explant with diameter approx. 3 to 10 mm. Shoot regeneration was performed from tuber discs, internodes and leaf explants using 6 different media. Different regeneration capacities were observed by the explants along 60 days. The average number of shoots was highest from tuber discs (6.2) than from leaf explants (2.6) which exceeds about three times; no shoot from internode explants cultured on the various media. Regenerated plantlets produced from both tuber discs and leaf explants exhibited random amplification of polymorphic DNA (RAPD) analysis using five selected primers to detect somaclonal variation. All the morphological variants were excluded. One of the regenerated plantlet derived from leaf-explants was true-to-type to the main *in vitro* plantlet, so it will be used as a source of explants for transformation experiments. The other regenerated plantlets derived from leaf explants and tuber discs show the presence and/or absence of polymorphic bands. Results also showed that microtubers were initiated on the etiolated shoots of the regenerants at the first 10 days. The etiolated shoots induced about 2.6 ± 0.6 and 2.2 ± 0.5 microtuber/explants.

Key words: *Solanum tuberosum* L., seed tuber, sprouting, micropropagation, microtubers, explants, regeneration, random amplification of polymorphic DNA (RAPD).

INTRODUCTION

Potato is one of the world's most important non-cereal food crops next to rice, wheat and corn in terms of total food production (Ross, 1986; Park et al., 1995). The tuber, the most important part of the plant, is an excellent source of carbohydrates, free essential amino acids particularly lycine, good quality protein, minerals and vitamins (FAO, 1984; Bajaj, 1987).

In vitro propagation of the potato by serial culture of axillary shoots (a leaf and its associated axillary bud) in separated nodes (Goodwin et al., 1980; Hussey and Stacey, 1981, 1984) is now becoming established as an effective means of rapidly multiplying new or existing cultivars in disease-free conditions.

Regeneration from cultured explants of potato is simpler than from protoplast and is applicable to a range of commercially grown cultivars. Plant regeneration from

*Corresponding author. E-mail: tarekmoussa@yahoo.com. Tel: +2 02 3567 6654 or +2 0100 153 1738.

Abbreviations: IAA, Indole-3-acetic acid; **NAA,** 1-naphthaleneacetic acid; **2,4-D,** 2,4-dichlorophenoxyacetic acid.

explant cultures is quicker and easier than protoplast culture (Wheeler et al., 1985). Potato have been shown to be easily regenerated both directly from organ cultures and indirectly via a callus phase under appropriate conditions, but their responses to published regeneration regimes have shown cultivar specificity (Wheeler et al., 1985; Hoekema et al., 1989). Although, many thousands of potato varieties are available around the world at the present time, cv. Desiree is considered to have high frequency regeneration on the greatest range of media than other cultivars tested (Wheeler et al., 1985; Yee et al., 2001).

In vitro production of microtubers by serial culture of individual nodes with axillary buds involves very complex developmental processes that include physiological, biochemical and structural changes being routinely used for disease-free seed production in potato (Vreugdenhil and Struik, 1989; Zaki, 1997; Gopal et al., 1998; Zhang et al., 2005a). Microtuber formation was localized at the stem base, in the leaf axil or at the place of stem branching. This process was more frequent in the cultures with a long subculture interval (3 to 4 months) and was genotypically specific (Novak and Zadina, 1987). The developmental steps necessary for the formation of tubers on potato plant were described by Vreugdenhil and Struik (1989); where axillary buds of the node form stolons with diageotropic shoot growth and reduced leaf growth.

It may be mentioned that environmental features implicated in microtuber induction are light and temperature, while medium components implicated in induction include sucrose, nitrogen, silver nitrate, growth regulators and natural products. Many substances have also been investigated including abscisic acid and the anti-gibberellin compounds or chemical growth retardants (alar, ancymidol, 2-chloroethyl trimethyl-ammonium chloride, coumarin, fluridone) or thidiazuron or antioxidant treatments (Hussey and Stacey, 1984; Tovar et al., 1985; Perl et al., 1991; Vreugdenhil et al., 1994; Kefi et al., 2000; Galal et al., 2002). Tuberization in potato was controlled by tuberonic acid and its glucosides which have a close relation with jasmonic acid in structure (Koda and Okazawa, 1988; Koda et al., 1988; Yoshihara et al., 1989). *In vitro* microtuberization provided an effective experimental model for physiological and metabolic mechanisms due to the similar developmental and structural features between tubers grown *in vivo* and *in vitro*. There is very few information about the screening of potential potato genotypes for salt tolerance as well as the effect of salinity on microtuber development, physiological changes and quality characteristics (Veramendi et al., 1999; Silva et al., 2001; Zhang et al., 2005b, 2006).

The purpose of this work was to compare a range of media and explants to select highly efficient reliable and reproducible regeneration system to maximize the production of regenerants for transformation of new genes to potato plant.

MATERIALS AND METHODS

Plant material

Virus–free seed tubers of potato (*Solanum tuberosum* cv. Desirée) were kindly provided from the Central Administration for Seed Certification, Agricultural Research Centre, Ministry of Agriculture and Land Reclamation, Giza, Egypt. The tubers were used as initial plant material.

Cultivation of seed-tuber

Tubers were brushed dry to remove mud, mechanical impurities and microorganisms. They were cleaned with water and soap for 30 min, washed thoroughly (5X) with running tap water for 15 min, then wrapped in dark paper bags and stored in a growth chamber at 18 ± 2°C (Zhang et al., 2005a, b) and 70% relative humidity. Rapid development of etiolated strong sprouts was encouraged by transfering the tuber every week from dark to indirect light (James et al., 1981; Merja and Marko, 1988). Under these conditions, the tuber sprouted after four weeks. The number of active eyes and the number of sprouts per eye were recorded after 30 days.

Sprouts culture

Sprouts of about 4 to 8 cm length, were cut carefully from the mother tubers and surface sterilized by 70% Clorox® (5.25% w/v sodium hypochlorite) for 20 min. Single node cuttings (with axillary bud) of the sterilized sprouts were implanted in 150 x 25 mm glass culture tubes (one node/tube) on multiplication medium, which consists of MS medium (Murashige and Skoog, 1962) containing vitamins, supplemented with additional B_5 vitamins (Gamborg et al., 1968), 3% sucrose, 100 ml/L myo-inositol and 0.7% agar. The pH of the medium was adjusted to 5.8 before autoclaving. After autoclaving, the medium was supplemented with 0.5 ml/L silver thiosulfate (STS) (Perl et al., 1988). All cultures were maintained in a temperature-controlled growth room at 18 ± 2°C with 16 h photoperiod and light intensity of 25 µmol/m²/s using white fluorescent lamps. After 4 weeks, the buds developed into plantlet having ~7 nodes. They were used as a source of nodal cuttings for micropropagation.

Micropropagation

Single node cuttings (with axillary bud and leaf), about 10 to 15 mm in length, of *in vitro* plantlets were routinely subcultured on 250 ml glass jars; each with 20 ml multiplication medium every 4 weeks. The pH of the medium was adjusted to 5.7 before autoclaving. The cultures were maintained in a temperature-controlled growth room at 18 ± 2°C with 16 h photoperiod and light intensity of 25 µmol/m²/s using white fluorescent lamps. The process was continued until complete plantlets were obtained in sufficient numbers (plantlets stock) and they were used as a source of different explants (internodes and leaves) for the regeneration experiment. The morphologically different plantlets (somaclones) were not used for subculturing.

Microtuberization

Single node cuttings (with axillary bud and leaf), about 10 to 15 mm in length, of *in vitro* micropropagated plantlets were cultured on 250

Table 1. The additives on MS medium with vitamins for shoot regeneration.

Chemical	M1	M2	M3	M4	M5	M6
Vitamins (ml/L)						
Glycine	—	—	2.0	—	—	—
Folic acid	—	—	0.5	—	—	—
D-Biotin	—	—	0.05	—	—	—
Growth regulators (ml/L)						
Indol-3- acetic acid (IAA)	1.0	0.4	—	2.0	—	—
1-Naphthalene acetic acid (NAA)	—	—	0.03	—	0.2	0.186
Gibberellic acid (GA_3)	10	0.4	0.5	1.0	0.02	5.0 / 5.0*
N6-Benzyladenine (BA)	1.0	—	3.0	3.0	1.0*	2.25/2.25*
Zeatin (ZN)	—	—	—	—	2.0/1.0*	—
Kinetin (KN)	—	0.8	—	—	—	—
Supplements (g/L)						
Casein hydrolysate	—	1.0	1.0	—	—	—
Sucrose	50.0	50.0	25.0	30.0	30.0	30.0
Agar	7.0	7.0	7.0	7.0	7.0	7.0

*The hormones are added to the successive medium.

ml glass jars; each with 20 ml fresh liquid medium using the method of Islam et al. (1999). This medium consists of liquid MS salts with vitamins, 80 g/L sucrose, 100 ml/L myo-inositol and 5 ml/L benzyl adenine (BA). The pH was adjusted to 5.7 before autoclaving. Five nodal cuttings were cultured in each jar (10 replicates each). The cultures were maintained in a temperature-controlled growth room at 20 ± 2°C and complete darkness. The cultures were examined weekly and then harvested after one month. The uniform microtubers were selected and used to form a stock, which were used as source of tuber discs for the regeneration experiment.

Regeneration

Tuber discs (8 to 10 mm diameter, 2 to 3 mm thickness), internodes without axillary buds (~8 to 10 mm) and leaf explants with central midrib (10 mm^2) were cultured in sterilized Petri-dishes (9 cm diameter) containing 6 different regeneration media to regenerate shoots. The media were developed by Tavazza et al. (1988) (M1), Alphonse et al. (1998) (M2), Jarret et al. (1980) (M3) and Yee et al. (2001) (M4) (as one step-procedure) as well as Moravčiková et al. (2003) (M5) and Wheeler et al. (1985) (M6) (as two-step procedure) (Table 1). The pH was adjusted to 5.7 before autoclaving.

Thirty explants (for internodes and leaves) and 18 explants (for tuber discs) were cultured. No special care was taken to separate microtuber pith from cortex and perimedullary portions due to the small size of the microtubers, so all the tuber discs contained both tissues. Petri-dishes were sealed with parafilm and incubated at 22 ± 2°C for one week in darkness followed by 16 h photoperiod under light intensity of 25 µmol/m^2/s using white fluorescent lamps for the remaining of incubation time. The Petri-dishes were examined after 7, 20, 40 and 60 days. The following indices were used for the organ formations according to Kikuta and Okazawa (1984):

Root formation index = (no. root formed × no. explant with roots)/(no. explant cultured)^2

Shoot-buds formation index = (no. shoot-buds formed × no. explant with shoot-buds)/(no. explants cultured)^2

Random amplification of polymorphic DNA (RAPD) analysis

DNA extraction

DNA isolation was performed using cetyl trimethylammonium bromide (CTAB) method of Doyle and Doyle (1990). 0.5 g fresh tissue was ground in liquid nitrogen, then suspended in 1m l preheated CTAB buffer. The suspension was incubated at 65°C for 60 min. The microfuge tube was inverted several times, then cooled to room temperature. The sample was then centrifuged at 1000 rpm for 10 min at -4°C and then the supernatant was transferred into a clean microfuge tube containing 0.5 ml chloroform:isoamyl (24:1). The sample was mixed by inverting gently the tube several times, and then centrifuged at 14000 rpm for 15 min at -4°C. The supernatant was transferred to a new tube and ice cold isopropanol (1000 µl) was added to precipitate the DNA. The tube was incubated at 20°C overnight, centrifugation was then carried out at 14000 rpm for 20 min at -4°C. The supernatant was discard and the pellet was washed carefully twice with 70% ethanol. The tube was allowed to air dry. 100 µl of sterile deionized distilled water was added to resuspend the DNA and then stored at 4°C.

DNA amplification and agarose gel electrophoresis

RAPD were performed as described by Williams et al. (1990) with minor modifications. Polymerase chain reaction (PCR) was performed in 25 µl reaction mixture each containing 0.5 U (0.25 µl) *Taq* DNA polymerase, 0.2 mM (2.5 µl) dNTPs, 5 µl (10X) reaction buffer, 20.4 ng (3 µl) genomic DNA and 5 pmole (3 µl) for each primer, which were completed with 9.25 µl sterile distilled water. The selected random primers used for RAPD as illustrated in Table 2.

The DNA amplification was performed using Biometra Uno thermal cycler programmed as follows: one cycle at 95°C for 3 min, 44 cycles at 92°C for 2 min, 37°C for 1 min and 72°C for 2 min and then one cycle at 72°C for 10 min for the last extension. The reaction was finally incubated at 4°C. The PCR amplification

Table 2. Selected random primers used in RAPD reaction.

Primer	Sequence (5'→3')	TM (°C)
B-01	GTTTCGCTCC	32
B-02	TGATCCCTGG	32
A-04	AATCGGGCTG	32
Z-01	TCTGTGCCAC	32
Z-04	AGGCTGTGCT	32

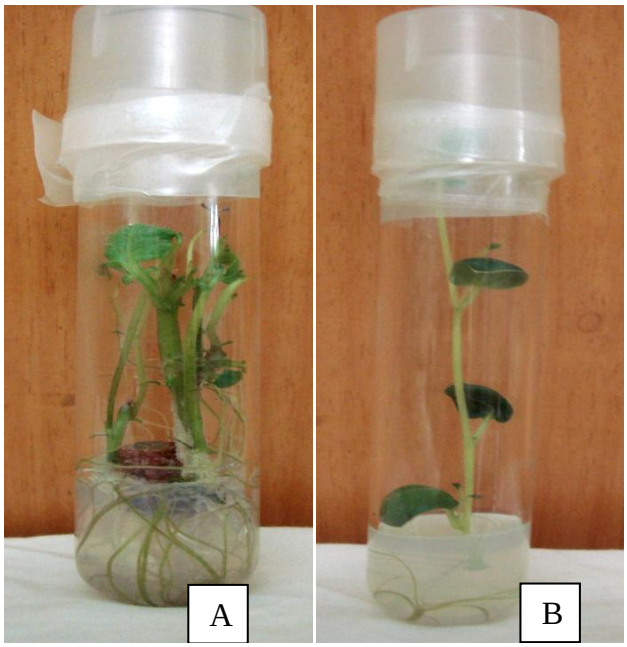

Figure 1. 4-week-old propagated plantlet from a single node cuttings of the sterilized sprouts [A] and the first *in vitro* plantlets (four-weeks old) [B].

products were separated by electrophoresis on 2% agarose gel in (1X) TAE buffer.

RESULTS

The seed tubers had six active eyes, which develop rapidly about two etiolated sprouts/eye when wrapped in dark paper bags and stored in a growth chamber at 18 ± 2°C with 70% relative humidity. After 4 weeks, sprouts of about 4-8 cm length were cut carefully from the mother seed tubers and surface sterilized by 70% Clorox® for 20 min. Single node cuttings were used as the main source of explants for the first *in vitro* plantlets (Figure 1A). The produced *in vitro* plantlets (7 to 8 cm in length) were used as a source of nodal cuttings for micropropagation (Figure 1B). These plantlets consisted of ~7 nodes, branched roots and trifoliate leaves as seen in Figure 2. All the morphologically variants were excluded from micropropagation.

The data presented in Table 3 and Figure 3 showed that the initiation of microtubers was asynchronous on etiolated shoots raised from the explants. Microtubers generally originate as aerial structures on the etiolated shoots, occasionally some microtubers were formed on emerged shoots in the media. Microtubers were initiated at the first 10 days. Microtubers were round-elliptical with pale yellow to reddish-brown in colour. The etiolated shoots induced about 1.32 average numbers of microtuber/explants with diameter ~3 to 10 mm at harvesting time (Figure 3).

Generally, on all the regenerated media, during the first week of incubation, tuber discs enlarged and the tuber skin ruptured. Calli initiated at the lower side of the discs adjacent to medium, vary in biomass, color and texture (Figure 4). The obtained results showed that the Jarret et al. (1980) medium gave the best results; few shoots per explant were observed after 20 days, which continue to grow forming multiple shoots at the end of the 60 days of cultivation. The shoots generated from the perimedullary portion at the lower side of the discs. All the regenerated shoots consisted of few nodes and green leaves. No roots were observed from the discs along the cultivation time (Table 4 and Figure 5). It may also be mentioned that Jarret et al. (1980) medium took less time to initiate shoots from tuber discs compared with other tested media. No shoots or roots were proliferated on medium of Wheeler et al. (1985) but only large mass of green callus were formed during the cultivation time. Other media Tavazza et al. (1988), Alphonse et al. (1998), Yee et al. (2001) and Moravčiková et al. (2003) showed the formation of few shoot per explant, which continued to elongate until the 60 days of cultivation. Only the shoots produced on Yee et al. (2001) and Moravčiková et al. (2003) media carried green leaves but those produced on Tavazza et al. (1988) and Alphonse et al. (1998) were bared (Table 4 and Figure 5).

Enlargement of internodes was initiated during the first week and slight proliferation of calli was initiated at the wounded edges of internodes on all the regeneration media but with variations in color and biomass (Figure 4). Swellings or protuberances calli continue to grow over the entire explants especially at the lower side adjacent to medium. On the Tavazza et al. (1988) and Alphonse et al. (1998) media, only few white and green calli were formed on the wounded sides as well as along the explants. No shoots or roots were observed on both media, while on Wheeler et al. (1985) medium, no shoots were produced but only few roots covered with dense white hairs were observed after the 20th day. Yellowish-white callus was grown and spread until the 60th day of cultivation. Shoots were regenerated on Jarret et al. (1980), Yee et al. (2001) and Moravčiková et al. (2003) media but with different regeneration capacities. Shoot primordium arose from the green calli at the side(s) of the explants. The Jarret et al. (1980) medium shows superiority by producing more shoot primordia per

Figure 2. Two to four weeks old plantlets with trifoliate leaves obtained from nodal cutting on micropropagation medium.

Table 3. Microtuberization of potato cv. Desirée on Islam et al. (1999) medium.

Parameter	Character
Time of initiation after cultivation	10 days
Average number of microtubers/explants	1.32 ± 0.4
Average number of microtubers/jar	6.6 ± 2.1
% tuberization/Jar	94%
Diameter of tubers (Ɵ mm)	3 - 10
Average weight of microtubers	0.2 - 0.5 g
Skin color of tubers	Pale yellow, Reddish-brown
Shape of tuber	Round to elliptical
Other observation	Asynchronous

Figure 3. Microtubers harvested from Islam et al. (1999) medium.

explant than the two other media (Table 5 and Figure 6).

During the first week on the different media, leaf explants were enlarged and upwardly rolled. Proliferation of calli (Figure 4), on all the regeneration media was performed at wounded edges of the explants during the first 20 days but with variations in color and biomass. Calli continue to grow till the 60 day of cultivation (Figure 7). Leaf explants cultured on Yee et al. (2001), produced both green calli and shoot primordia from those calli at day 20. By time the calli continue to grow especially at

Figure 4. Tuber disc (left), internodes (middle) and leaf (right) explant after 7 days on regeneration media.

Table 4. Regeneration from tuber discs on six different media for 20, 40 and 60 days of cultivation.

Media	Day	Total no. shoots	Average no. of explant	Average no. shoots/shooted explant	Index of shoot-bud formation
	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
M1	40	5.0 ± 0.0	0.3 ± 0.6	1.3 ± 0.5	0.02 ± 0.0
	60	5.0 ± 0.0	0.3 ± 0.6	1.3 ± 0.5	0.02 ± 0.0
	20	6.0 ± 0.0	0.4 ± 0.6	1.2 ± 0.5	0.02 ± 0.0
M2	40	6.0 ± 0.0	0.4 ± 0.61	1.20 ± 0.5	0.02 ± 0.0
	60	6.0 ± 0.0	0.4 ± 0.6	1.20 ± 0.5	0.02 ± 0.0
	20	9.0 ± 0.0	0.5 ± 0.6	1.1 ± 0.4	0.1 ± 0.0
M3	40	22.0 ± 0.0	1.1 ± 1.2	2.2 ± 0.7	0.2 ± 0.0
	60	49.0 ± 0.0	2.7 ± 3.4	6.1 ± 1.9	0.30 ± 0.0
	20	6.0 ± 0.0	0.3 ± 0.6	1.2 ± 0.5	0.02 ± 0.0
M4	40	6.0 ± 0.0	0.3 ± 0.6	1.2 ± 0.5	0.02 ± 0.0
	60	6.0 ± 0.0	0.3 ± 0.6	1.20 ± 0.5	0.02 ± 0.0
	20	4.0 ± 0.0	0.2 ± 0.4	1.0 ± 0.0	0.01 ± 0.0
M5	40	4.0 ± 0.0	0.2 ± 0.4	1.0 ± 0.0	0.01 ± 0.0
	60	4.0 ± 0.0	0.2 ± 0.4	1.0 ± 0.0	0.01 ± 0.0
	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
M6	40	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	60	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

M1, Tavazza et al. (1988); M2, Alphonse et al. (1998); M3, Jarret et al. (1980); M4, Yee et al. (2001); M5, Moravčiková et al. (2003); M6, Wheeler et al. (1985). Data are the means of 18 tuber discs explants ±SD.

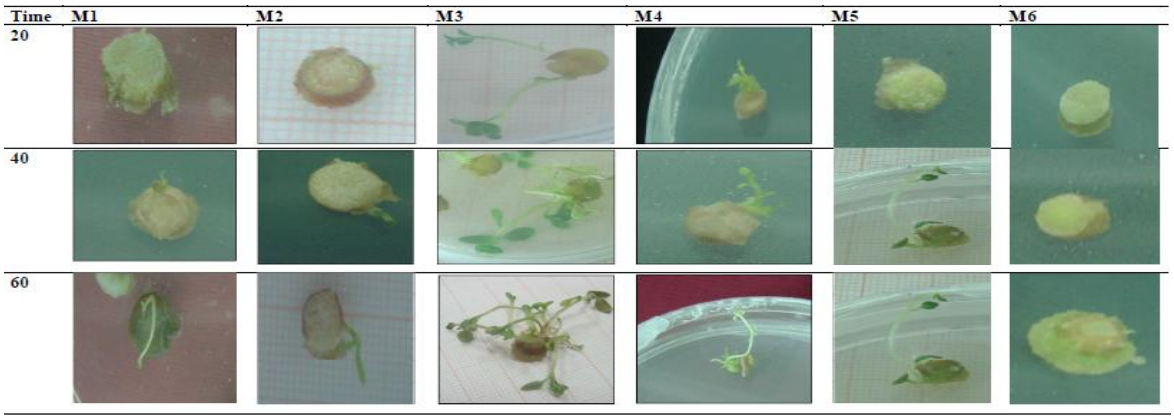

Figure 5. The morphogenic responses of tuber discs when cultured on six different regeneration media. M1, Tavazza et al. (1988); M2, Alphonse et al. (1998); M3, Jarret et al. (1980); M4, Yee et al. (2001); M5, Moravčiková et al. (2003); M6, Wheeler et al. (1985).

Table 5. Regeneration from internodes explants on six different media for 20, 40 and 60 days of cultivation.

Media	Days	Total no. shoots	Average no. explant with shoots	Average no. shoots/shooted explant	Index of shoot-buds formation	Total no. roots	Average no. explant with roots	Average no. roots/rooted explant	Index of roots formation
M1	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	40	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	60	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
M2	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	40	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	60	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
M3	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	40	18.0 ± 0.0	0.6 ± 1.0	1.8 ± 0.9	0.1 ± 0.02	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	60	18.0 ± 0.0	0.6 ± 1.0	1.8 ± 0.9	0.1 ± 0.02	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
M4	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	40	10.0 ± 0.0	0.4 ± 0.6	1.3 ± 0.5	0.02 ± 0.02	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	60	10.0 ± 0.0	0.4 ± 0.6	1.3 ± 0.5	0.02 ± 0.02	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
M5	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	40	7. 0 ± 0.0	0.2 ± 0.4	1.0 ± 0.0	0.01 ± 0.01	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	60	7.0 ± 0.0	0.2 ± 0.4	1.0 ± 0.0	0.01 ± 0.01	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
M6	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	40	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	24.0 ± 0.0	1.1 ± 1.1	1.6 ± 0.9	0.1 ± 0.02
	60	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	24.0 ± 0.0	1.1 ± 1.1	1.6 ± 0.9	0.1 ± 0.02

M1, Tavazza et al. (1988); M2, Alphonse et al. (1998); M3, Jarret et al. (1980); M4, Yee et al. (2001); M5, Moravčiková et al. (2003); M6, Wheeler et al. (1985). Data are the means of 18 tuber discs explants ±SD.

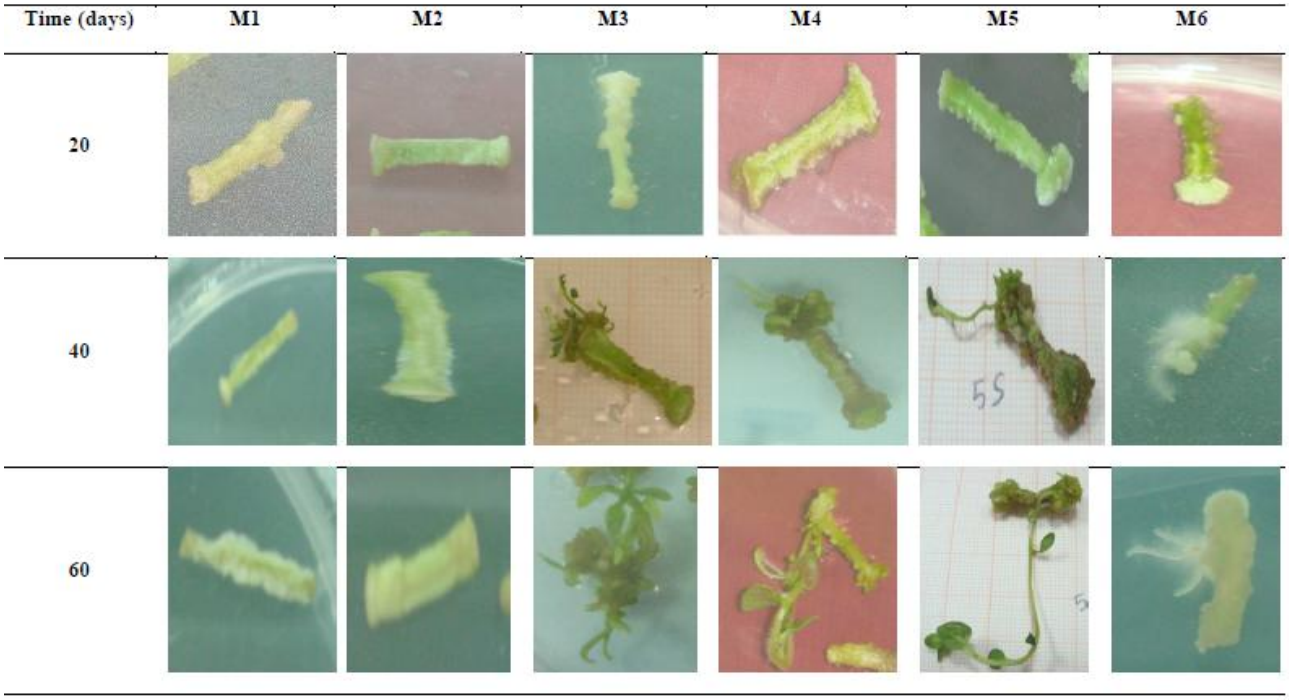

Figure 6. The morphogenic responses of internode explants when cultured on six different regeneration media. M1, Tavazza et al. (1988); M2, Alphonse et al. (1998); M3, Jarret et al. (1980); M4, Yee et al. (2001); M5, Moravčiková et al. (2003); M6, Wheeler et al. (1985).

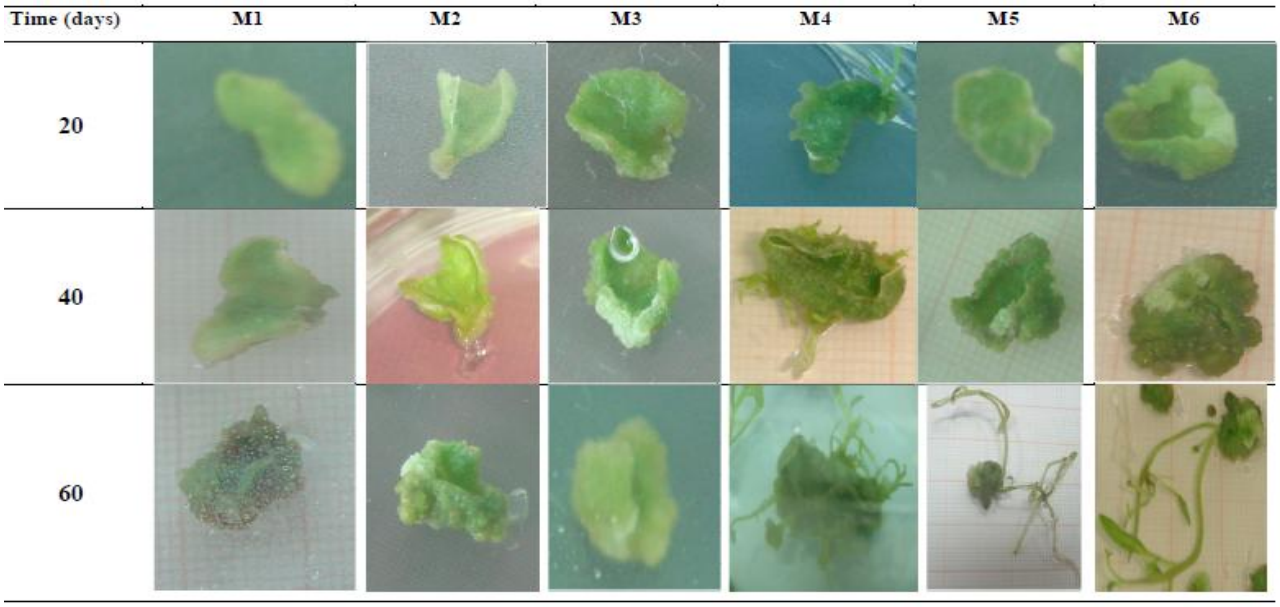

Figure 7. The morphogenic responses of leaf explants when cultured on six different regeneration media. M1, Tavazza et al. (1988); M2, Alphonse et al. (1998); M3, Jarret et al. (1980); M4, Yee et al. (2001); M5, Moravčiková et al. (2003); M6, Wheeler et al. (1985).

the lower side of the rolled leaf faced to the medium forming numerous swellings or protuberances calli. Multiple shoot primordia arose from those calli-structures, which elongate by time and carry numerous leaves at the end of the cultivation time. It may also be mentioned that medium of Yee et al. (2001) took less time to initi shoots from explants compared to the other tested media. On the other hand, on the medium of Moravčiková et al. (2003) and that of Wheeler et al. (1985), few shoots per explant arose from calli during the 40^{th} to 60^{th} days of cultivation; the shoots produced from the later carry green leaves as recorded (Table 6 and Figure 7).

Based on the previously mentioned results, multiple shoot regeneration from tuber discs and internodes were better on Jarret et al. (1980) medium but Yee et al. (2001) medium was better in case of leaf explants; internodes explants cultured on the various media did not induced the profuse shoot formation seen from tuber discs and leaf explants. It could be concluded from (Tables 4 to 6 and Figures 4 to 7) that among all the previous regeneration media (M1→M6), the M3 medium (Jarret et al. (1980) medium) was found to be the best medium for multiple shoot regeneration from tuber discs (6.13 average number of shoots) and M4 medium [Yee et al. (2001) medium] was found to be the best medium for multiple shoot regeneration from leaf explants (2.6 average number of shoots) so, both media were used in transformation experiments.

Regenerated plantlets produced from both tuber discs and leaf explants exhibited RAPD analysis using 5 selected primers to detect somaclonal variation (Figures 8 to 12). All the morphological variants were excluded. Regenerated plantlet derived from leaf-explants (lane 3) was true-to-type to the main *in vitro* plantlet (lane 6), so could be used as a source of explants for transformation experiments. The other regenerated plantlets derived from leaf explants (lanes 1, 2, 4, 5) and those derived from tuber discs show the presence and/or absence of polymorphic bands.

Results showed that the initiation of microtubers was asynchronous on etiolated shoots raised from the explants. Microtubers were initiated on the etiolated shoots of the regenerants at the first 10 days. Microtubers were round-elliptical with pale yellow colour. The etiolated shoots induced about 2.6 and 2.2 average numbers of microtuber/explants (Table 7 and Figure 13).

DISCUSSION AND CONCLUSION

Greater demand for plants especially for the purpose of food and medicine is one of the causes of their rapid depletion from primary habitats (Boro et al., 1998). Micropropagation has been proved to be very efficient technique to speed-up the production of high quality pathogen-free plantlets, in terms of genetic and physiological uniformities, with high photosynthetic potential (Sathish et al., 2011; Supaibulwattana et al., 2011).

This work focus on the *in vitro* propagation of potato cv. Desireé as a source of explants (nodal cuttings, leaves, internodes and tubers) for *in vitro* experiments. Well-developed propagated shoots *in vitro* were initiated from single-nodal cuttings, of *in vitro* plantlets on MS media

Table 6. Regeneration from leaf explants on six different media for 20, 40 and 60 days of cultivation.

Media	Day	Total no. shoot	Average no. explant	Average no. shoots/shooted explant	Index of shoot-buds formation
M1	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	40	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	60	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
M2	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	40	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	60	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
M3	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	40	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	60	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
M4	20	10.0 ± 0.0	0.3 ± 0.7	1.4 ± 0.5	0.02 ± 0.001
	40	20.0 ± 0.0	1.2 ± 1.3	2.4 ± 0.5	0.1 ± 0.1
	60	36.0 ± 0.0	1.2 ± 1.5	2.6 ± 1.2	0.1 ± 0.01
M5	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	40	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	60	8.0 ± 0.0	0.2 ± 0.6	1.5 ± 0.6	0.01 ± 0.003
M6	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	40	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	60	7.0 ± 0.0	0.2 ± 0.6	1.8 ± 0.5	0.01 ± 0.002

M1, Tavazza et al. (1988); M2, Alphonse et al. (1998); M3, Jarret et al. (1980); M4, Yee et al. (2001); M5, Moravčiková et al. (2003); M6, Wheeler et al. (1985). Data are the means of 18 tuber discs explants ±SD.

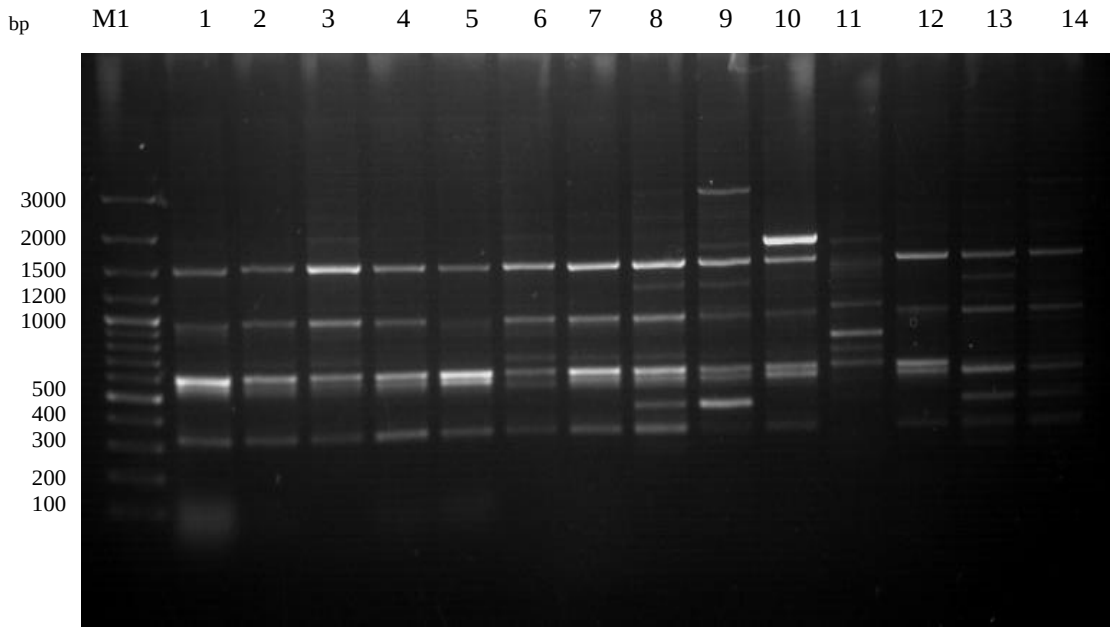

Figure 8. RAPD profiles of *in vitro* plantlet and regenerated plantlets using preselected random primer (Z4). M1:3000 bp DNA ladder; lane 6 is the *in vitro* plantlet (control), the lanes 1 to 5 were the regenerated plantlets from leaf explants and the lanes 7 to 14 were the regenerated plantlets from tuber discs.

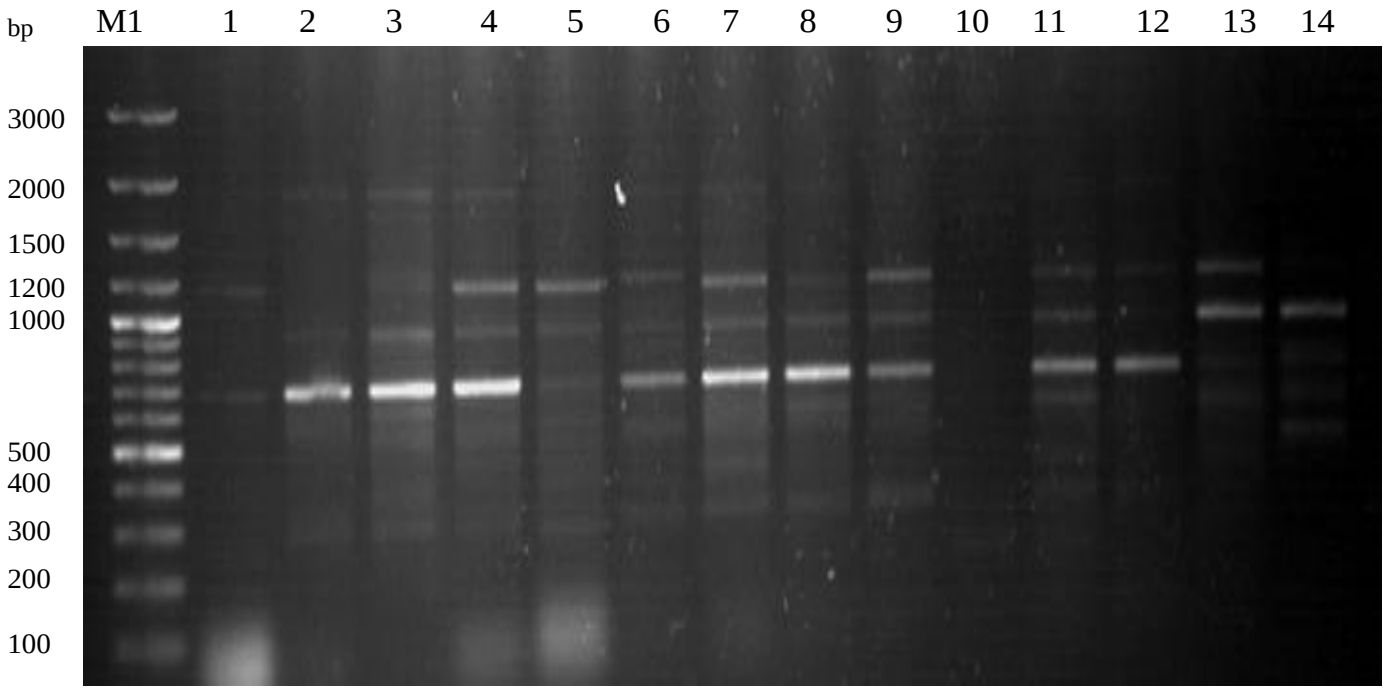

Figure 9. RAPD profiles of *in vitro* plantlet and regenerated plantlets using preselected random primer (B2). M1:3000 bp DNA ladder; lane 6 is the *in vitro* plantlet (control), the lanes 1 to 5 were the regenerated plantlets from leaf explants and the lanes 7 to 14 were the regenerated plantlets from tuber discs.

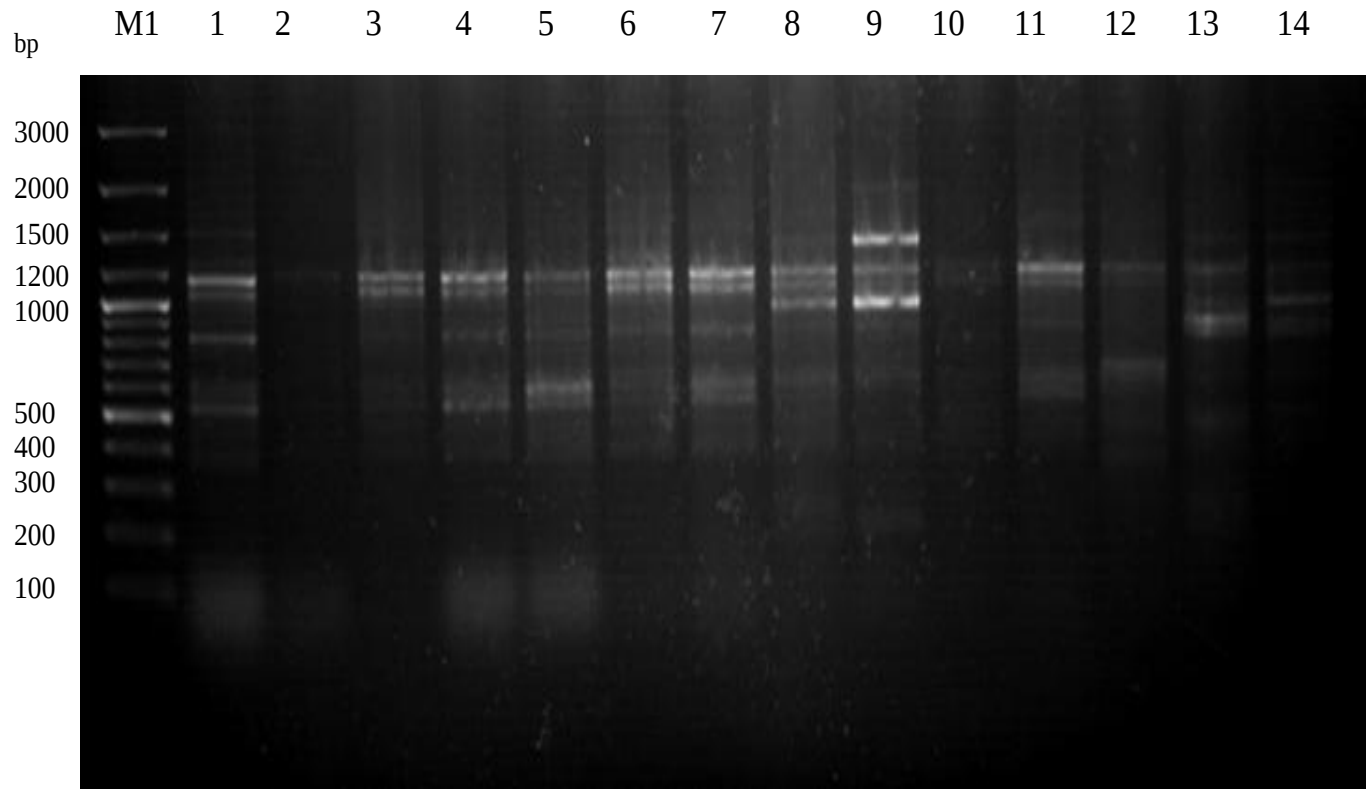

Figure 10. RAPD profiles of *in vitro* plantlet and regenerated plantlets using preselected random primer (Z1). M1:3000 bp DNA ladder; lane 6 is the *in vitro* plantlet (control), the lanes 1 to 5 were the regenerated plantlets from leaf explants and the lanes 7 to 14 were the regenerated plantlets from tuber discs.

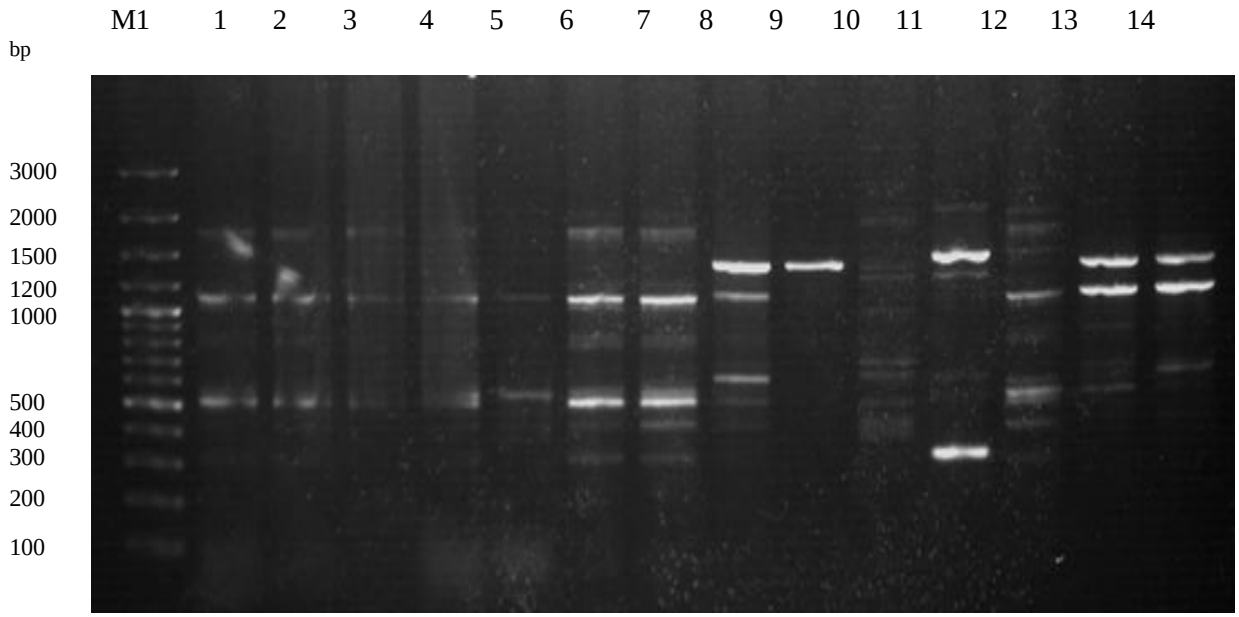

Figure 11. RAPD profiles of *in vitro* plantlet and regenerated plantlets using preselected random primer (A4). M1:3000 bp DNA ladder; lane 6 is the *in vitro* plantlet (control), the lanes 1 to 5 were the regenerated plantlets from leaf explants and the lanes 7 to 14 were the regenerated plantlets from tuber discs.

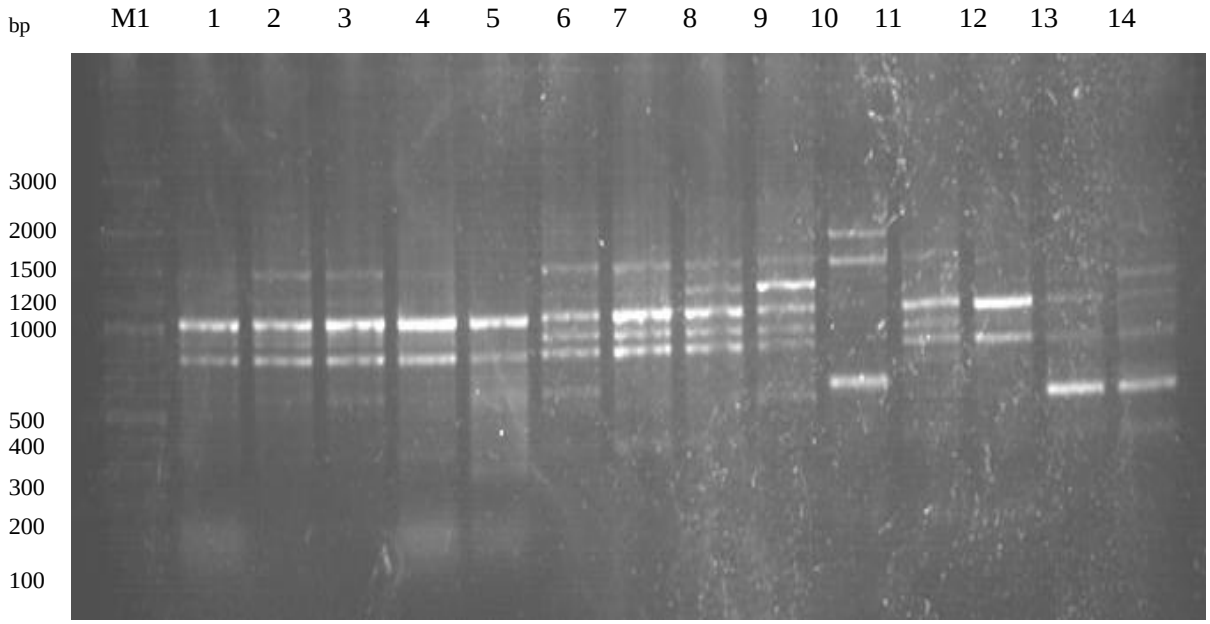

Figure 12. RAPD profiles of *in vitro* plantlet and regenerated plantlets using preselected random primer (B1). M1:3000 bp DNA ladder; lane 6 is the *in vitro* plantlet (control), the lanes 1 to 5 were the regenerated plantlets from leaf explants and the lanes 7 to 14 were the regenerated plantlets from tuber discs.

containing vitamins, supplemented with 3% sucrose, 100 ml/L myo-inositol, 0.5 ml/L silver thiosulfate (STS). Roots were developed at the base of the explant on the same medium. The use of single-node cuttings excised from tissue cultured plantlets is more common and avoids the influence of tuber tissue from which sprout sections originate (Hussey and Stacey, 1981; Levy et al., 1993; Seabrook et al., 1993; Leclerc et al., 1994; Mohamed and Alsadon, 2010). It was also used for long-term storage of plant materials in isolation as an important part of breeding as well as germplasm conservation (Novak and Zadina, 1987). Following surface sterilization, nodal

Table 7. Microtuberization of true-to-type regenerants derived from tuber discs and leaf explants on Islam et al. (1999) medium.

Parameter	Regenerant from tuber disc	Regenerant from leaf explant
Average number of microtubers (per explant)	2.6 ± 0.6	2.2 ± 0.5
Average number of microtubers (per jar)	13.7 ± 1.5	11.7 ± 1.5
Skin color of tubers	Pale yellow	Pale yellow
Shape of tuber	Round to elliptical	Round to elliptical
Other observation	Asynchronous	Asynchronous

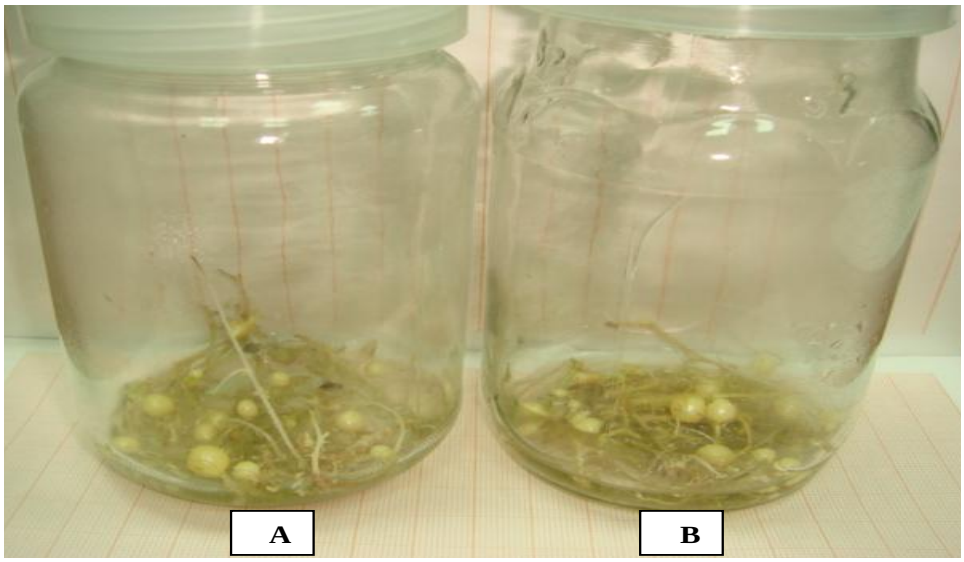

Figure 13. Microtubers from regenerated shoots derived from tuber discs (A) and leaf explants (B) harvested from Islam et al. (1999) medium.

cuttings are grown intensively on standard agar or in liquid propagation medium, in the absence of growth regulators, or with low levels of cytokinin then the cultured shoots rapidly develop strong branched roots at the plantlet base (Hussey and Stacey, 1981; Novak and Zadina, 1987).

Murashige and Skoog medium (MS) is the mostly used medium for rapid growth of cell, tissues and organ of plants. Macro- and micro-nutrient constituents employed in tissue culture media may exercise a profound effect on metabolism (Narayanaswamy, 1994). Myo-inositol is routinely added to the culture medium in small amounts (100 ml/L). It plays a role in many biosynthetic pathways, and improves cell growth (Narayanaswamy, 1994). The medium requires to be supplemented by vitamins of the B-complex [Thiamine HCl (B_1), Nicotinic acid (B_3), Pyridoxin-HCl (B_6)] to enhance healthy growth of tissues in culture. Vitamins, either individually or as a mixture, play a catalytic role in cell metabolism apart from being a factor in accessory food supply, but their requirements vary from species to species (Narayanaswamy, 1994).

Sugars are added to the growing media as a source of carbon (Murashige and Skoog, 1962; Jones, 1988). Potato tissues *in vitro* are generally not autonomous for photosynthesis and frequently depend on a source of organic carbon such as sucrose (George, 1986). Sucrose levels 2 to 3% were commonly used for micropropagation (Forti et al., 1991).

Many reporters routinely use silver thiosulphate (STS) during plant growth and maintenance of *in vitro* potato plants to promotes growth and provides significantly larger leaves than would otherwise be the case (Perl et al., 1988; Chang and Chan, 1991; Hulme et al., 1992), which might imply either that these cultivars produce a lot of ethylene or that they are particularly susceptible to it (Hulme et al., 1992).

Temperatures of 20 to 25°C promote micropropagated plantlet growth (Akita and Takayama, 1994a, b; Leclerc et al., 1994). Photoperiod, irradiance, and light spectral quality can be used to control the growth of potato plantlets *in vitro*, thereby in some instances, avoiding the use of growth regulators, which could possibly cause off-types (Seabrook et al., 1993; Wilson et al., 1993; Seabrook and Douglass, 1998). Spectral quality of light is the relative intensity and quantity of the different wavelengths emitted by a light source and perceived by photoreceptors within the plant. Cool-white or Grolux fluorescent lamps are commonly used for potato tissue

culture (Schilde-Rentschler and Schmiediche, 1984; Lindsay, 1987; Tao et al., 1987). 16-h light period were recommended for optimal growth and the maintenance of vegetative growth of potato plantlets *in vitro* (Dodds et al., 1992; Jao and Fang, 2004a, b). Seabrook (2005) reported that in a medium lacking growth regulator, roots form readily on potato plantlets *in vitro* which was similar to our results.

In this work, well-defined tuberization were performed *in vitro* by culturing nodal cuttings from the stock of *in vitro* plantlets, *in vitro* selected and transformed lines, when cultured on liquid MS medium (pH 5.8) supplemented with 5 ml/L BA and 8% sucrose. The culture was incubated at 20 ± 2°C for 4 weeks in complete darkness.

In vitro tuberization in potato is influenced by many factors including carbohydrate supply, light, photoperiod, temperature, gibberellins (GA), nutrition, genotype and but hormone played a dominant role in this process (Arteca, 1996; Momoh et al., 2002; Zhang et al., 2005a, b; Banerjee et al., 2006; Agud et al., 2010; Altindal and Karadogan, 2010; Hoque, 2010). Much attention has so far been focused on the use of cytokinins such as BA (Rosell et al., 1987; Lentini and Earle, 1991).

Wan et al. (1994) induce tuberization in hydroponically cultured potato plants by lowering the pH from 5.8 to 5.2 when the tuber started to develop, and that the MS medium pH drifts to acidity with time to 3.5 to 4.0, the possibility of pH itself being directly related to tuberization was considered.

As a general rule, photoperiod is a major morphogenetic control of *in vitro* tuberization in potato (Seabrook et al., 1993; Coleman and Coleman, 2000). It should be mentioned that microtuberization efficiency increased when micropropagated source plants were grown under long day (16/8 h day/night) followed by continuous darkness (Seabrook et al., 1993). This encourage Jackson (1999) to suggest that it is actually the length of the dark period rather than the light period that is important for tuberization and so, potato plantlets *in vitro* can be used to produce microtubers by promoting effect of complete darkness either as continuous (Nowak and Asiedu, 1992; Dobranszki and Mandis, 1993) or periodical regime (Garner and Blake, 1989; Lentini and Earle, 1991; Seabrook et al., 1993; Struik and Wiersema, 1999).

In the present study, the produced microtubers were round-elliptical with pale yellow to reddish-brown in colour. In this context, it may be mentioned that the microtubers induced in continuous darkness are white or yellow or even brown, whereas those induced under light-dark photoperiod are green (Gopal and Minocha, 1997). Temperature is generally lowered (15 to 20°C) for microtuber induction and affected both microtuber number and fresh weights (Leclerc et al., 1994; Akita and Takayama, 1994a, b).

Carbohydrates, especially sucrose, appeared to influence tuberization, not only as substrates for the biosynthesis of starch, but also as factors controlling morphogenesis (Hussey and Stacey, 1984; Vreugdenhil et al., 1998; Xu et al., 1998). The major flux in potato tuber carbon metabolism was the conversion of sucrose through hexose phosphates to starch (Fernie et al., 2002). The use of a higher concentration of sucrose is recommended as it promotes microtuberization (Hussey and Stacey, 1984; Garner and Blake, 1989; Vreugdenhil and Helder, 1992; Gopal et al., 1998), and thus would produce more microtubers of bigger size. In this respect, the use of 8% sucrose induced the initiation of tubers, gave more and large microtubers, compared to lower concentrations (Garner and Blake, 1989; Khuri and Moorby, 1996).

Exogenous cytokinins supplementation, especially BA, to the standard MS medium containing high concentration of sucrose promote potato tuberization and are considered to be tuber-inducing factors (Gopal et al., 1998; Pelacho et al., 1999; Rodrigues-otubo et al., 1999; Teisson and Alvard, 1999). Cytokinin causes stolon formation *in vitro* followed by tuberization (Forsline and Langille, 1976; Mauk and Langille, 1978). Starch accumulation required for tuber initiation and development is strengthened by the finding that cytokinins influence starch formation by their inhibitory effect on amylase activity (Sanz et al., 1996).

In this work, six different media from previous studies and 3 types of explants (internode, tuber disc and leaf) obtained from *in vitro* plantlets were used for this investigation. The explants were incubated at 22 ± 2°C for a week in darkness followed by 16 h photoperiod for the rest of the incubation time.

In this context, many investigations have been published on the *de novo* organ formation and regeneration of plantlets or shoots from explants of different origins (root, hypocotyl, stem and leaf) of *Solanum tuberosum* (Wheeler et al., 1985; Visser et al., 1989; Hulme et al., 1992; Park et al., 1995; Cearley and Bolyard, 1997; Khatun et al., 2003).

Depending upon the genotype, the origin and type of the explant and the culture conditions, it is often necessary to alter the composition and /or concentration of growth regulators in the culture medium (Kut et al., 1984). Generally, a low ratio of auxin to cytokinin is required for adventitious shoot development.

In this work, All the regenerated media consisted mainly of basal MS medium with addition of 100 mgl^{-1} myo-inositol, 30 gl^{-1} sucrose, auxin, cytokinins, GA3 and 0.5 ml/L STS. The explants were incubated at 22 ± 2°C for a week in darkness followed by 16 h photoperiod under light intensity of 25 $\mu mol/m^2/s$ using white fluorescent lamps for the remain incubation time.

Webb et al. (1983) stated that MS media was suitable for 6 cultivars of potato and the shoot formation was shown to depend on the hormonal composition of the medium employed as well as genotype used. De Block

(1988) noticed that the callus formation on B_5 medium was similar to that on MS medium, but calli on MS medium regenerated faster and poduced more shoots than those on B_5 medium.

The regeneration protocol involving an auxin and cytokinin pulse (Hovenkamp-Hermelink et al., 1988) combined with the use of silver ions is a superior method to those published for a number of potato cultivars. Silver thiosulphate (STS) has been showed to increase regeneration rates of a number of cultivars (Perl et al., 1988; Hulme et al., 1992). Other researchers do not require the addition of STS either in the regeneration or transformation systems (Park et al., 1995). Tuber discs regenerated into shoots needed to grow on a medium containing different growth regulators in addition to casein hydrolyate, which has a sterols and amino acids contents (Jarret et al., 1980; Indrayanto et al., 1995).

The success of organ induction was influenced by various factors including the interaction between endogenous and externally applied growth regulators added to the culture medium (Taylor and Veilleux, 1992). The differentiation of culture tissue depends on the ratio of auxin to cytokinin in the culture medium whereas low auxin: cytokinin ratios stimulate the formation of shoots (Akiyoshi et al., 1983). Incorporation of cytokinin affected the level of endogenous auxin by inhibiting the oxidation of additional IAA, maintaining the optimum level of this auxin for shoot morphogenetic response (Antis and Northcote, 1973; Manjula and Nair, 2002). Benzylaminopurine (BAP), zeatin or kinetin added individually to the nutrient medium might stimulate shoot formation. In terms of effectiveness in promoting shoot initiation, reports were contradictory; showing zeatin to be less, equal, or often superior to BA as the cytokinin component (Webb et al., 1983; Iapichino et al., 1991; Anjum and Ali, 2004a, b), while kinetin, although stimulating some shoot development (Padmanabhan et al., 1974) is usually the least effective.

Auxins and cytokinins also mediated the morphogenetic effects of light on potato *in vitro* (Sergeeva et al., 1994). Morphogenesis of potato tissue cultures can be manipulated by light regimes as photoperiod, irradiance, and light spectral quality (Seabrook et al., 1993; Wilson et al., 1993; Seabrook and Douglass, 1998).

GA3 activates the cell division cycle by regulating the transition from G1 to S phase, and G2 to M phase by the expression of several cyclin-dependent protein kinases (CDKs), which lead to an increase in mitotic activity in the intercalary meristem (Fabian et al., 2000).

In the present investigation, swellings or protuberanches calli (node-like-structure) were produced at or near the wound site of all the explants within a week after incubating in complete darkness then continue to grow over the entire explants especially at the lower side adjacent to medium. Initial dark treatment has been found to be beneficial for plant regeneration of potato (Park et al., 1995).

As previously mentioned in our results, M3 medium (Jarret et al., 1980) was found to be the best medium for multiple shoot regeneration from tuber discs (6.1 average number of shoots) and M4 medium (Yee et al., 2001) medium was found to be the best medium for multiple shoot regeneration from leaf explants (2.6 average number of shoots). This may be due to their nutritional and/or their hormonal compositions and ratios.

In the present study, the shoots or roots generate mainly from the callus that appear at the lower side of the explants after 20 days of cultivation in case of tuber explants and 40 days in case of leaf and internodes explants. Localized areas of rapid cell division leads to the formation of external parenchymatous tissues (meristemoids) on the explants, either from the epidermal cells, the sub-epidermal cells (Handro et al., 1972) or the epidermal cells only (Bigot, 1971), which gives rise to shoot primordials. This process required about 35 days in potato tuber explants. The expression of morphogenetic competence from tuber explants is dependent on the explant source, nutrient medium, and environmental growth conditions (Jarret et al., 1980). Regeneration from leaf explants of potato cv. Desireé can be divided into 3 phases; the initiation of callus, the initiation of shoots on this callus and shoot development (Wheeler et al., 1985). Internodes from the *in vitro* shoots (like that of sprouts) give rise either to roots or to shoots, but never both. The internodes of the *in vitro* shoots show a low percentage of regenerated shoots. The percentage of regenerated roots, on the contrary, is quite high (Quraishi et al., 1987). It has also been observed that best results concerning rhizogenesis are shown by internodes placed in inverse polarity, and on the medium in which the concentration of BA is the lowest (Quraishi et al., 1987). A relatively low concentration of auxin in the medium is required for root formation on the culture. Indole-3-acetic acid (IAA) is markedly superior to any other auxin in root forming activity. 1-Naphthaleneacetic acid (NAA) is somewhat less effective than IAA, and 2,4-dichlorophenoxyacetic acid (2,4-D) shows no stimulation on root formation (Okazawa et al., 1967).

In this study, it was noticed that the single-step protocols of M3 and M4 media yielded the highest regeneration frequency over the other single-step protocols of M1 and M2 media as well as the two-step protocols of M5 and M6 media. In addition, our results revealed that multiple shoot regeneration from tuber discs were better on M3 medium but M4 medium was better in case of leaf explants. Internodes explants cultured on the various media did not induced the profuse shoot formation seen from tuber discs and leaf explants, but only calli and/or roots were observed. Evidence indicated that it is very difficult to generalize the method of inducing adventitious shoots and regeneration *in vitro* because the process of organogenesis of potato may vary among species, cultivars (clones) and especially the donor tissue

(Webb et al., 1983; Hulme et al., 1992; Dale and Hampson, 1995; Anjum and Ali, 2004a, b). The cultivar Desireé was used in the present study since it displayed a high capacity for regeneration (Wheeler et al., 1985) and for transformation, and so it has been used extensively for potato transformation (Dale and McPartlan, 1992). Genotypic differences for regeneration ability from explant cultures of potato clones have also been reported by Wheeler et al. (1985), Cardi et al. (1992) and M'Ribu and Veilleux (1990). Due to their morphological differences, the explants do not represent identical tissues and therefore a direct comparison of explants (tuber and stem) may not be appropriate (Anjum and Ali, 2004b). The potato tuber can not be treated as a homogeneous mass of tissue when cultured *in vitro*. Consideration must be given to the diversity of tissue types which exist in potato tuber (Reeve et al., 1970). It is, however, important that in potato, various tissues can be used as explants for shoot generation directly (Anjum and Ali, 2004b).

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