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Morphological and Molecular Characterization of Potato Microtubers Production on Coumarin Inducing Medium

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ABSTRACT

Shoot cultures of potato microtubers (Spunta cultivar) were established from meristem explants growing on Murashige and Skoog (MS) medium supplemented with 0.04 mg L^{-1} kinetin (Kin) + 1 mg L^{-1} Indole acetic acid (IAA). The results revealed that sucrose is an important factor for micro-tubers formation. The highest tuber formation was achieved when 12% sucrose was added to culture medium. Among three tested levels of coumarin, 20 and 40 mg/L were more effective regardless of sucrose concentration. Concerning the potential of combination of sucrose and coumarin the highest percentage of tuberization (86.7%) was recorded with 6% sucrose + 20 mg L^{-1} coumarin. The highest number of microtubers per plantlet was detected when 9% sucrose was added to coumarin - induced medium. For molecular analysis, results refer that although microtubers-derived plants of potato expressed a high degree of polymorphism relatively to field-grown source, they were genetically similar. Slight molecular genetic differences were detected in plantlets derived from micro-tubers.

Key Word: Potato; Micro-tubers; Sucrose; Coumarin; RAPD analysis

INTRODUCTION

Due to its high nutrative value, potato is cultivated worldwide in the temperate and subtropical zones. It became a stable food in developing and industrial countries, while it is used for additional purposes. Potato is an annual, herbaceous plant, which is vegetatively propagated by the tuber. In traditional seed potato production system, it is mainly propagated via seed tubers. This method has disadvantages in terms of poor seed health and low rate of multiplication (Beukema & Van Der Zaag, 1990). It can also be propagated rapidly in large scale by tissue culture techniques. Recently, seed production system has been designed with a phase of *in vitro* multiplication through either plantlet or microtuber production. Such systems allow faster multiplication and the build-up of a large stock, which is disease-free. *In vitro* plantlets usually produced from nodal cuttings and micro-tubers are small *in vitro* tubers, which can be produced all year round on complete plantlets or on plant organs (Ranalli, 1997). Microtubers have some advantages over *in vitro* plantlets. They are very convenient and easy to transport and store, material is available and production is possible all year round with little bench space, they have the same health status as *in vitro* plantlets, whereas it is not necessary to produce just before use (Struik & Wiersema, 1999). Extensive physiological research has revealed that tuberization is controlled by several factors. Tuberization initiation is also associated with hormonal changes in the plant (Vreugdenhil & Struik, 1989). Several workers have focused their attention on the application of exogenous growth regulators for stimulating *in vitro* tuberization (Coleman *et al*., 2001; Tugrul & Samanci, 2001). Growth regulators are commonly used in *in vitro* multiplication, both during the production of plantlets and in microtuber production. Coumarin is highly effective as a tuberization promoter (Radouani, 1997). A concentration of 25 mg/L was optimum for tuberization stimulation and the optimum temperature for coumarin activity is between 20 to 25ºC (Prange *et al*., 1990). Coumarin is also affected by the concentration of sugar in the nutrient medium. Activity is reduced if the percentage of sugar is lower than 4% or higher than 10% (Roca *et al*., 1978).

Genetic variability generated during tissue culture is defined as somaclonal variation (Larkin & Scowcroft, 1981). Tissue cultured plants lose totipotency with length of time in culture, which is probably due to loss or mutation of genes that are essential for plant regeneration (Scowcroft, 1984). Molecular analysis to detect possible variation of plant cvs. Monalisa and Spunta derived from conventional tubers, microtubers, cell cultures and micro-plants, was carried out by Mandolino *et al*. (1996). Analysis of the genetic stability was performed by probe hybridization and genomic southern blots (restriction fragment length polymorphismor or RFLP) and by randomly amplified polymorphic DNA (RAPD) analysis. The results show for both cultivars indicated the identity of the DNA patterns of *in vitro* tuber deriving plants with the pattern of the conventional tuber-deriving plant. By contrast, the pattern of identification of cv. Spunta was greatly altered when DNA was extracted from two-years-old suspension cultures growing in the presence of growth regulators. This investigation aimed to study the microtuberization process and morphological characters of microtubers of potato produced *in vitro* as affected by combinations of sucrose

and coumarin and molecular analysis for their genetic identification.

MATERIALS AND METHODS

Establishment of aseptic tissue cultures. Certified tubers of potato cv. Spunta were used as plant material. Tubers were sprouted in dark at room temprature (25 ± 2ºC). When the sprouts had 3 - 5 nodes (4 - 5 cm length), they were removed from the tuber and surface sterilized by 10% (v/v) clorox (contained 5.25% of sodium hypochlorite) for 20 min, then rinsed three times in sterile distilled water. Under binocular, meristem tips excised in length about 0.5 mm were placed into tubes (7.5 × 2.5 cm), which contained 10 mL of MS medium Murashige and Skoog (1962) supplemented with 0.04 mg L^{-1} Kin and 1 mg L^{-1} IAA. Medium was solidified with 6 g L^{-1} agar. Sub-culturing of meristem to a fresh medium was carried out every months. Indexing of potato viruses, i.e., PVX, PVY and PLRV in obtained plantlets, was carried out by ELISA test (Clark & Adams, 1977).

Micro-propagation of plantlets. Meristem-derived virus-free plantlets (6 - 9 cm height) were used as mother source for rapid multiplication. Plantlet was cut into single node cutting, each having an axillary bud. Immediately after preparation, cuttings were then aseptically placed horizontally onto surface of agar solidified medium in 250 mL jars, each containing 40 mL medium and sealed with autoclaved plastic covers. The medium used was basal MS-medium supplemented with 0.04 mg L^{-1} Kin + 1 mg L^{-1} IAA + 3% sucrose and 0.7% agar. Cultures were incubated in a growth chamber under the same conditions as mentioned before. After four weeks, plantlets with 6 - 7 nodes were used for subculture. Single cuttings propagation process was repeated four times at four weeks intervals to increase the number of plantlets.

***In vitro* tuberization.** Shoots (6 - 7 nodes) resulted from the previous step were cultured into liquied MS medium supplemented with a factorial combinations of 5 sucrose, 4 coumarin concentrations plus 2 mg L^{-1} Kin, pH was adjusted to 5.7 before autoclaving for 25 min at 121ºC. Each culture jar (250 mL) received 40 mL medium and contained 5 plantlets. Cultures were incubated under complete dark conditions at 18 - 20ºC. Data of micro-tubers number/tuberized plantlet, percentage of tuberization, microtuber fresh weight, number of days to reach 50% tuberization and tuberization rate were recorded after 8 weeks of culturing on the tuberization medium.

Molecular analysis. Genomic DNA was isolated on a mini-prep scale as mentioned by Murray and Thomposon (1980). Small pieces (0.5 g) of leaf tissue of micro-tuber-derived plants were frozen in liquid nitrogen in Eppendorf tubes and homogenized in 500 µL of extraction buffer (2% CTAB, 1.4 m NaCl, 20 mm EDTA pH 8.0, 100 mm Tris-HCl, pH 8.0, 0.1 m ß-Mercatoethanol). The extract was incubated at 60ºC for 20 min. To this, 500 µL phenol: chloroform: isoamyl alcohol (24:24:1) were added and mixed by vortexing for 30 sec followed by centrifugation at 10,000 xg for 5 min at room temperature and aqueous phase was transferred to another tube. This was once again extracted with 500 µl chloroform: isoamyl alcohol (24:1) in Eppendorf tube. To the aqueous phase, 0.6 volume of isopropanol was added, genomic DNA was precipitated and the fibrous genomic DNA was spooled. Genomic DNA was then washed three times with 70% ethanol, vacuum dried, dissolved in TE containing 10 mg mL^{-1} RNase and incubated at 37ºC for 30 min, followed by extraction with phenol: chloroform: isoamyl alcohol (24: 24:1) and the aqueous phase was transferred to a fresh tube. Thereafter, the genomic DNA was precipitated by adding 0.3 m sodium acetate, pH 5.2 (final concentration) and 2.5 vol of ethanol and collected by centrifugation at 10,000 *g* for 20 min at 4ºC. The pellet was washed with 70% ethanol, vacuum dried and dissolved in TE. Six random 10-base oligonucleotide primers (Operon technologies Inc., Alameda, California) were designed for use in RAPD analysis. Each primer was 10 bp lengths. The primers are OPK01 (5' TGC CGA GCT G 3'), OPK02 (5' GTG AGG CGT G 3') OPK03 (5' CCC TAC CGA C 3'), OPK04 (5' TCG TTC CGC A 3'), OPK06 (5' TCG TTC CGC A 3') and OPK07(5'CCA CAG CAG T 3'). PCR reactions were carried out in 50 µL volumes containing 100 mg of genomic DNA, 1.0 µm of each primer, 200 µm dNTPs, 10 mm Tris-HCl, pH 8.3, 50 mm KCl, 1.5 mm MgCl2 and 0.001% gelatine. The Taq-DNA polymerase concentration was 1.5 units per assay. Reaction was conducted in a Perkin Elmer 2400 thermo cycler (Germany). Mashine was programmed for 45 cycles as follows: 94ºC/5 min (1 cycle), 94ºC/30 sec, 36ºC/30 sec, 72ºC/2 min (44 cycles) and 72ºC/7 min (1 cycle), then held at 4ºC. The amplification products were size-separated by gel electrophoresis in 1% agrose gels with 1 x TBE buffer using a Pharmacia (GN. 100) submarine gel electrophoresis apparatus and stained with ethidium bromide and visualized with UV transilluminator and photographed. A 100-bp DNA ladder (Promega) was used as a DNA standard with molecular sizes of 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp. DICE computer package was used to analyze RAPD-PCR products (Yang & Quiros, 1993).

Statistical analysis. A randomized complete block design was used in a factorial arrangement; 4 concentrations of coumarin; 5 concentrations of sucrose with 3 replicates each. Data were analyzed according to the method described by Snedecor and Cochran (1967).

RESULTS AND DISCUSSION

***In vitro* microtuber formation.** The carbon source in the medium is a very important factor for *in vitro* tuberization. Carbon directly influences carbohydrate synthesis. In tuber crops, there is an inverse relationship between shoot and tuber growth. However, the dry matter distribution pattern varies between the different tuber crops. In potato, shoot

growth virtually stops once tuber initiation has taken place. Carbohydrate translocation to the tuber only takes place once need of the shoots has been fulfilled. Our finding (presented in Table I & II) indicated that the sucrose-free medium is non-inducing regardless of the presence of coumarin since no tuber is formed during the observation period (8 weeks). Otherwise, our data indicated that sucrose concentration is very important factor for tuberization process and characteristics of obtained tubers. The highest value of tuberization (81.8%) of coumarin-free was registered when 12% sucrose was used. At the same level of sucrose, 2.7 micro-tubers plantlet^{-1}, were formed and needed less than 15 days to give 50% tuberization (Table I). Otherwise, 3% sucrose in inducing tuberization medium gave heavier micro-tubers (410.5 mg) compared with the other treatments and gave 42.1% of obtained tubers weighted over 500 mg in agreement with results obtained by Lawrence and Barker (1963). They found no tuber formation with low sucrose concentration (0 - 2%), slow development of tubers with 3, 6 and 9% sucrose and rapid initiation with 12%. Also, Fung *et al.* (1972) reported that 12% sucrose promotes tuberization by virtue of its presence as a carbon source and not because of its attendant elevated osmotic concentration. Accordingly, as the level of carbon source increases, the *in vitro* tuberization process is fastened with this respect sucrose concentration used for *in vitro* tuberization of potato varies from 2 to 12%. Plamer and Barker (1972) used 2% sucrose only in the medium and

Table I. Tuberization of potato on MS medium supplemented with various combinations of sucrose and coumarin

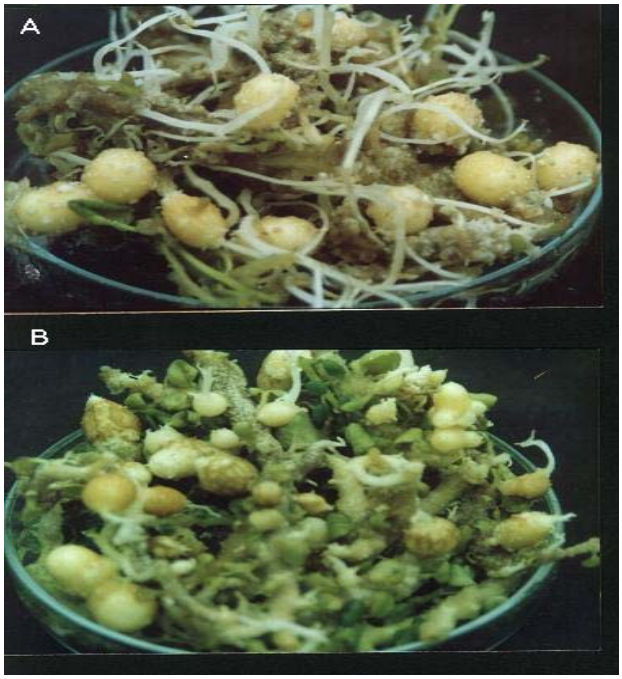
Treatments	Tuberization (%)	Microtubers per tuberized plantlet	Days to reach 50% tuberization	Average fresh weight (mg)
1	0.0	0.0	0.0	0.0
2	0.0	0.0	0.0	0.0
3	0.0	0.0	0.0	0.0
4	0.0	0.0	0.0	0.0
5	80.0	1.6 ± 0.0 09	15	410.5 ± 5.50
6	80.0	1.2 ± 0.0 07	49-56	89.1 ± 6.80
7	73.3	1.4 ± 0.0 05	49-56	54.5 ± 3.30
8	20.0	0.7 ± 0.010	56<	380.5 ± 13.80
9	66.7	2.7 ± 0.020	>15	323.3 ± 9.50
10	86.7	2.3 ± 0.009	15-21	472.0 ± 8.10
11	80.0	3.4 ± 0.025	42	111.6 ± 5.90
12	27.8	1.3 ± 0.008	56<	51.6 ± 3.50
13	73.3	2.5 ± 0.010	15-21	153.6 ± 5.80
14	68.0	2.1 ± 0.004	28-35	246.6 ± 10.00
15	50.0	3.7 ± 0.006	42	82.1 ± 4.50
16	50.0	1.7 ± 0.003	21	271.5 ± 8.50
17	81.8	2.7 ± 0.008	>15	190.4 ± 5.00
18	33.3	2.0 ± 0.006	56<	16.0 ± 2.00
19	27.3	1.6 ± 0.002	56<	16.0 ± 2.80
20	16.7	1.0 ± 0.004	56<	42.4 ± 3.50

Mean ± SE

Table II. Tuberization rate of potato plantlets growing on medium contained the combinations of sucrose and coumarin

Treatments		Rate of Tuberization (%)						
Source%	mg Cumarin/l	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks	7 weeks	8 weeks
0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0	20	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	60	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3	0	53.3	60.0	66.7	66.7	66.7	73.3	80.0
3	20	0.0	0.0	0.0	10.0	20.0	40.0	80.0
3	40	0.0	0.0	0.0	0.0	0.0	30.0	73.3
3	60	20.0	20.0	20.0	20.0	20.0	20.0	20.0
6	0	66.7	66.7	66.7	66.7	66.7	66.7	66.7
6	20	33.3	66.7	80.0	80.0	86.7	86.7	86.7
6	40	20.0	20.0	20.0	30.0	50.0	60.0	80.0
6	60	5.6	11.1	11.1	11.1	11.1	17.0	27.8
9	0	20.0	53.3	73.3	73.3	73.3	73.3	73.3
9	20	20.0	32.0	36.0	60.0	68.0	68.0	68.0
9	40	16.7	33.3	33.3	33.3	50.0	50.0	50.0
9	60	33.3	50.0	50.0	50.0	50.0	50.0	50.0
12	0	68.1	77.3	77.3	81.8	81.8	81.8	81.8
12	20	27.3	27.3	27.3	27.3	27.3	27.3	33.3
12	40	0.0	0.0	16.7	16.7	16.7	16.7	27.3
12	60	26.7	26.7	33.3	33.3	33.3	33.3	16.7

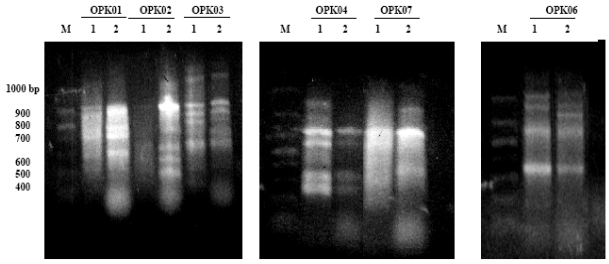
Fig. 1A. Tuberization of potato plantlets using formed on medium contained 6% sucrose + 20 mg/L coumarin, (B) The highest frequency of micro-tuber per explant formed on medium contained 9% sucrose + 40 mg/L coumarin



reported that the cytokinin concentration affected the *in vitro* tuberization even at low sucrose concentration. However, Koda and Okazawa (1983) used medium supplemented with 4% sucrose in combination with cytokinins.

Coumarin is one of phenolic compounds, which have several physiological effects on plant cells. It considered being primarily a plant growth inhibiting compound. Moreover, coumarin has an active effect in tuberization of potato stem segments *in vitro*. In this investigation, three levels, i.e., 20, 40, and 60 mg L^{-1} of coumarin added to culture medium was examined for their potential on *in vitro* tuberization of potato. The data of Table I revealed that coumarin at 20 and 40 mg L^{-1} are more effective on tuberization compared with 60 mg L^{-1} coumarin regardless of the sucrose concentration. Percentage of tuberization was higher at 20 and 40 mg L^{-1} than that obtained by 60 mg L^{-1} coumarin with different levels of sucrose added to culture medium. Data on the role of sucrose in combination with coumarin on tuberization indicated that 6% sucrose and 20 mg L^{-1} coumarin was optimum since percent tuberization was 86.7 followed by 6% sucrose and 40 mg L^{-1} coumarin, which registered 80.0% tuberization of cultures (Table I, Fig. 1A). The highest number of microtubers per plantlet was registered when 9% sucrose was added to coumarin-induced medium (Table I, Fig. 1B). However, addition of 12% sucrose to coumarin-induced medium delayed tuberization and needed more than 56 days to give 50% of

Fig. 2. RAPD-PCR amplification products of micro-tubers derived plants (1) and tuber field grown plants (2). M referred to DNA marker with 100 bp ladder and OPK 01, 02, 03, 04, 06 and 07 are primers with 10-mer



micro-tubers at all coumarin concentrations (Table I). Concerning the tuberization curve, using of 12% sucrose alone gave the highest value of earlier tuber formation. This treatment registered 68.1% tuberization within the first two weeks followed by 6% sucrose (Table II). However, the highest rate of tuberization during the eight weeks of culturing was observed when medium contained 6% sucrose plus 20 mg L^{-1} coumarin. The obtained results are in accordance with those reported by Stalknecht (1972). In his study, the effect of coumarin on *in vitro* tuberization of potato explants indicated, no tuberization occurred at 1 mg L^{-1} coumarin and in the control lacking coumarin; however, tuberization was 30% at a concentration of 10 mg L^{-1} coumarin. Coumarin at 25 to 50 mg L^{-1} was optimum for the initiation of tubers. 100 mg L^{-1} coumarin, the tuberization process was delayed and the tubers were small compared with those under the treatments of 25 and 50 mg L^{-1}. Moreover, tubers formed in the coumarin-treated cultures were larger than those grown in the medium with kinetin (2.5 mg L^{-1}). In the same direction, Stallknecht and Farnsworth (1982b) mentioned that coumarin stimulates tuberization of excised axillary shoots of potato sprouts cultured *in vitro.*

Molecular characterization. RAPD fingerprints have recently been used to study the somaclonal variation in tissue culture process. In our study, RAPD was performed in two different source of DNA, i.e., tissue culture-derived microtubers and field growing tubers. DNAs of both were prepared from leaves and amplified by PCR using six random oligonucleotide primers. Amplification products were separated by agrose gel elctrophorosis to reveal band polymorphism. A total of 61 different DNA bands were reproducibly obtained, 14.8% of which were polymorphic as shown in Tables III and IV. Otherwise, data presented in Tables III, IV and Fig. 2 show that each primer produced between 3 - 9 amplification products that ranged in size between approximately 411 and 1200 bp. The genetic differences that detected in tissue culture-derived micro-tubers versus field grown tubers were low across all primer used (OPK01, OPK02, OPK03, OPK04, OPK06 & OPK07) (Fig. 2). Micro-tubers derived plants of potato expressed a

Table III. Amplified fragments obtained from the DNA of field grown-derived tubers and *in vitro* derived micro-tubers of potato via RAPD-PCR

Primer	Size of the bands(bp)	Source of tubers	
		(In Vitro)	(Field)
OPK01	918	1	1
	869	1	1
	760	0	1
	711	1	1
	600	1	1
Total		4	5
OPK02	1127	0	1
	918	1	1
	760	0	1
	641	1	1
	571	1	1
	511	1	1
Total		4	6
OPK03	1200	1	1
	1184	1	1
	918	1	1
	869	1	1
	789	1	1
	711	1	1
	461	1	1
	571	1	0
	511	1	0
Total		9	7
OPK04	966	1	0
	853	1	0
	759	1	1
	693	1	1
	517	1	1
	469	1	1
Total		6	4
OPK06	1088	1	1
	1013	1	1
	944	0	1
	839	1	1
	638	1	1
Total		4	5
OPK07	966	0	1
	788	1	1
	722	1	1
	576	1	1
Total		3	4

Table IV. Primers with aribitrary sequence tested for their effectiveness in the RAPD analysis that produced plymorphic bands

Primer	Number of bands in the gel		Size of the polymorphic bands (bp)	% of polymorphic
	Total	Polymorphic		
OPK01	9	1	760	11.1
OPK02	10	2	1127 760	20.0
OPK03	16	2	571 511	12.5
OPK04	10	2	966 853	20.0
OPK06	9	1	944	11.1
OPK07	7	1	966	14.3

high degree of polymorphism relative to field-grown source cultivars with the six primers tested Fig. 2. In conclusion, high value of genetic similarity, were obsereved between derived tuber plant and derived microtuber plant and our results demonstrate that the RAPD technique can be successfully applied to determine the genetic fidelity of micropropagated potato plants. In this respect, marker analysis has been successfully used to study the degree of genetic changes in plants regenerated *in vitro* such as pea (Cecchini *et al*., 1992), sugarbeet (Sabir *et al*., 1992) and wheat (Brown *et al*., 1993). Saker *et al*. (2000) mentioned no significant variation in *in vitro* regenerates of date palm. RAPD analysis showed genetic variation in only 4% of analyzed tissue culture derived plantlets, which were incubated for 6 - 12 months under 25˚C.

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