In vitro propagation, microtuberization, and molecular characterization of three potato cultivars

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Abstract

Sprouts of potato tubers were excised from the three potato cultivars Agria, Hermes, and Spunta, sterilized and subjected to shoot formation and propagation on Murashige and Skoog (MS) medium supplemented with 1 mg dm^{-3} 6-benzylaminopurine (BAP) + 0.5 mg dm^{-3} gibberellic acid. Shoots were rooted on MS medium supplemented with 1 mg dm^{-3} indole-3-butyric acid. To increase shoot vigour prior tuber formation, shoots were subcultured on MS medium supplemented with 0.56 mg dm^{-3} BAP, 0.11 mg dm^{-3} 2,4-dichlorophenoxyacetic acid, and 0.96 mg dm^{-3} naphthaleneacetic acid. Under dark, microtuberization on MS media supplemented with 4 mg dm^{-3} of both BAP and kinetin was better than 4 mg dm^{-3} BAP alone, where they induced higher number of microtubers per shoot and/or the percentage of shoots that formed microtubers. The highest frequency of microtuber formation was achieved when sucrose at high concentration (8 %) was used as carbon source in culture media. Glucose ranked at the second position whereas fructose reduced the microtuber formation frequency when it was used alone or in combination with glucose. Under the applied culture conditions, cvs. Agria and Hermes showed better micropropagation and microtuberization in comparison to cv. Spunta. In addition, isozyme and RAPD techniques revealed that Agria and Hermes are closer to each other when compared with the third cultivar.

Additional key words: auxins, cytokinins, gene expression, micropropagation, RAPD, *Solanum tuberosum.*

Introduction

Potato (*Solanum tuberosum* L.) is one of the most important crops worldwide (Jones 1994). It is vegetatively propagated using pieces or whole potato tubers, but a virus infection causes crop reduction to almost a half or even one third, which varies from place to place and from season to another (Anoop and Chauhan 2009). Micropropagation becomes the alternative to conventional propagation of potatoes and includes two main steps: multiplication and acclimatization of the *in vitro* obtained plants to growing under field conditions. The previous reports indicated that induction of regenerants on cultured explants is cultivar dependent (Yee *et al*. 2001, Saker *et al*. 2012). Microtuberization can be used to solve the problems of transplanting the plantlets from *in vitro* to *in vivo* conditions (Wang and Hu 1982, Mashhadi and Moeini 2015). Microtubers are used for growing disease-free potatoes in many countries (Wang and Hu 1982, Chaudhary and Mittal 2014).

Microtuberization in potato needs the right interaction between several factors including rather low temperatures (15 - 20 °C) and high sucrose and cytokinin concentrations (Wang and Hu 1982, Al-Hussaini *et al*. 2015). These interactions seem to be genetically determined (Ranalli 2007, Nistor *et al*. 2010). Influence of genotypes on *in vitro* propagation and production of microtubers in potato was investigated by Ranalli *et al*. (1994) and Nistor *et al*. (2010). While microtubers usually have a fresh mass ranging from 24 to 273 mg (Ranalli 2007), they have similar morphological and biochemical characteristics compared to field produced tubers. Consequently, microtubers are the recommended plant materials in several applications, including exchange of plant materials, germplasm conservation, plant metabolism research, and genetic transformation (Rosell *et al*. 1987, Tabori *et al*. 1999).

Polymorphism in isozyme electrophoretic patterns and DNA markers were used for the characterization of cultivars in several plant species, which reflects their genetic diversity (Badr *et al.* 2002, Mustafa *et al*. 2005, El-Kholy *et al*. 2006, El-Shazly and El-Mutairi 2006,

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Abbreviations: BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; EST - esterase; GA_3 - gibberellic acid; IBA - indole-3-butyric acid; IPOX - indophenol oxidase; KIN - kinetin; MS - Murashige and Skoog; MVSP - multi-variate statistical package; NAA - naphthaleneacetic acid; POX - peroxidase; RAPD - random amplified polymorphic DNA.

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Kumar *et al*. 2010, 2014, Khalil 2013). Isozymes and DNA markers were applied to detect genetic variation in potato (Pedro and Carlos 1995) as well as in other plant species (Hammad 2009).

In potato, the application of *in vitro* multiplication, microtuberization, and application of molecular markers were previously applied (Estrada *et al.* 1986, Demeke *et al*. 1996, Kaur *et al.* 2000, Coloman *et* al. 2001, Sakha *et al.* 2004, Liljana *et al.* 2012), but information about the coordination between the data of these techniques is not sufficient. Consequently, the aim of this study is to apply conditions suitable for multiplication and micro-tuberization of potato *in vitro*, and to clear up the effect of genotype on these processes.

Materials and methods

Tubers of three potato (*Solanum tuberosum* L.) cultivars, Agria, Hermes and Spunta were obtained from the Sohag Agricultural Center, Sohag, Egypt. Tubers were incubated at a temperature of 30 °C and a relative humidity of 70 % for two months in order to sprout. The sprouts were cut, sterilized by immersion in 5 % (v/v) sodium hypochlorite for 6 min and in 0.1 % (m/v) $HgCl_2$ for 1 min and washed 3 times in sterile distilled water, 10 min each. They were separately cultured in 100-cm^3 glass jars containing so called shoot multiplication medium: Murashige and Skoog (1962; MS) medium supplemented with 1 mg dm^{-3} 6-benzylaminopurine (BAP), 0.5 mg dm^{-3} gibberellic acid (GA_3), 30 g dm^{-3} sucrose, and 8 g dm^{-3} agar (pH 5.8). Cultures were incubated at 25 ± 2 ºC, a 16-h photoperiod, and an irradiance of 100 μmol m^{-2} s^{-1}. Three replicates of each cultivar were prepared and number of shoots per sprout, length of shoot, and number of nodes per shoot were determined after one month.

For shoot multiplication, nodal segments (about 0.8 cm length) of *in vitro* grown shoots were cultured on MS medium supplemented with different concentrations of BAP and GA_3 as indicated in Table 2. Forty explants were cultured in four jars for each treatment and incubated under culture room conditions. Frequency of shoot formation, number of shoots per explant, length of shoot and fresh mass of shoot clusters were determined.

For induction of callus formation, internode, node, and shoot tip explants (each of about 4 mm length) of the three potato cultivars were cultured on MS basal medium supplemented with 0.56 mg dm^{-3} BAP, 0.11 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.96 mg dm^{-3} naphthaleneacetic acid (NAA) for one month. Forty explants of each explant type were cultured in four jars and incubated under culture room conditions. After one month incubation, fresh mass of callus was determined.

Vigorous potato shoots for microtuberization were obtained when shoot tips or nodal cuttings were cultured on MS medium supplemented with 0.56 mg dm^{-3} BAP, 0.11 mg dm^{-3} 2,4-D and 0.96 mg dm^{-3} NAA (shoot growth medium). Comparison between growth of potato shoots on shoot multiplication medium and shoot growth medium was fulfilled where cultures were incubated for 40 d under culture room conditions. Number of shoots/explant, length of shoot, number of nodes/shoot and fresh mass of shoot were determined.

Potato microshoots (3 - 4 cm long) were cultured on full strength MS medium supplemented with different concentrations of indole-3-butyric acid (IBA; 0, 0.5, 1, 2, and 3 mg dm^{-3}). Forty microshoots of Agria and Hermes cultivars and thirty five of Spunta were cultured in four jars for each treatment. Cultures were incubated in the culture room for one month. Frequency of root formation, number of roots per a microshoot, and length of roots were determined. The plantlets of the three potato cultivars were transferred to soil after hardening for four weeks under laboratory conditions.

In vitro regenerated shoots of cv. Hermes were cut and transferred to MS basal medium supplemented with 4 mg dm^{-3} BAP and 80 g dm^{-3} sucrose. Cultures were incubated at 20 °C for two months either in continuous darkness (46 microshoots) or at a 16-h photoperiod (48 microshoots). Frequency of microtuber production, number of microtubers per shoot, and fresh mass of each microtuber were determined.

In order to determine the effect of BAP in combination with kinetin (KIN) as well as irradiance on microtuber production from the three potato cultivars, microshoots were cultured on MS basal medium supplemented with 80 g dm^{-3} sucrose and 4 mg dm^{-3} BAP or 4 mg dm^{-3} BAP + 4 mg dm^{-3} KIN (microtuber formation media). Cultures were incubated at 20 °C either under dark for 8 weeks or for two weeks in a 16-h photoperiod followed by 6 weeks in darkness. Four replicates of each treatment were prepared. Frequency of microtuber production, number of microtubers per shoot, and fresh mass of each microtuber were registered.

The effect of sugar type on microtuber production of the three potato cultivars was also examined. Microshoots of the three cultivars were subcultured on microtuber formation medium supplemented with 80 g dm^{-3} sucrose, 80 g dm^{-3} glucose, 80 g dm^{-3} fructose or glucose + fructose (40 g dm^{-3} of each). Cultures were incubated at 20 °C and 16-h photoperiod for two weeks followed by 6 weeks in continuous darkness. Four replicates were prepared for each treatment. Frequency of microtuber production, number of microtubers per shoot and fresh mass of each microtuber were determined.

For isozymes analysis, potato shoot cuttings of the three potato cultivars were grown for one month on shoot growth medium at a 16-h photoperiod and transferred to induce microtuber formation on three types of media (shoot multiplication, shoot growth, and microtuber formation media) at 20 ºC under dark for 15 d. Then microshoots were collected, washed from agar and subjected to native protein extraction. One gram of a

plant material was ground in 1 cm^3 of extraction buffer (0.1 μM Tris-HCl, pH 7.0, and 0.002 M cystein) in a mortar at 4 ºC. The homogenate was centrifuged at 13 148 *g* and 4 ºC for 15 min. Supernatants were collected for immediate electrophoresis in 7.5 % (m/v) polyacrylamide slab gels. Gels were run at 18 mA for 6 h at 10 ºC in 0.025 M Tris-base + 0.192 M glycine buffer (pH 8.9). Peroxidase, indophenol oxidase, and esterase were stained according to previous protocols (Brewer 1970).

For RAPD analysis, DNA was extracted from microshoots of the three potato cultivars using the modified cetyltrimethylammonium bromide protocol described by Porebski *et al.* (1997). Eleven random 10-mer primers (OPA-02, OPA-03, OPA-04, OPA-05, OPA-08, OPA-13, OPC-02, OPE-02, OPK-02, OPP-13 and OPaT-8) were used for DNA amplification. The polymerase chain reactions (PCR) were carried out in 25 mm^3 volume containing 50 ng of genomic DNA template, 30 pmol mm^{-3} primers, 0.2 μM each of dATP, dCTP, dGTP and dTTP, 10× buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, and 2 units of *Taq* polymerase (*AB* gene)]. Amplification was performed in a *Perkin-Elmer/GeneAmp*® PCR system *9700* (*PE Applied Biosystems*, Foster City, USA) programmed to fulfill 35 cycles after an initial denaturation cycle at 94 ºC for 5 min. Each cycle consisted of a denaturation step at 94 ºC for 45 s, an annealing step at 36 ºC for 50 s, and an elongation step at 72 ºC for 1 min. The primer extension segment was extended to 7 min at 72 ºC in the final cycle. The amplification products were resolved by electrophoresis in a 1.5 % (m/v) agarose gel containing ethidium bromide (0.5 μg cm^{-3}) in 1× TBE (750 mM Tris-HCl, 900 mM boric acid, and 2 mM Na_2-EDTA) buffer at 99 V. The electrophoretic DNA banding patterns obtained from the three tested potato plants of the three cultivars were analyzed by the *MVSP* computer software program of Nei and Li (1979).

Mean number of the cultured samples (microshoots, plantlets, or microtubers) in three jars were calculated and subjected to statistical analysis. Data were presented as means ± standard deviations (SDs) according to the method described by Snedecor and Cochran (1980). Analysis of variance (*ANOVA*) was performed using the software *SPSS 16*. The level of significance was measured running a Tukey test; $P \leq 0.05$ was considered as significant.

Results and discussion

After one month of culture on MS medium supplemented with 1 mg dm^{-3} BAP + 0.5 mg dm^{-3} GA_3, sprout explants formed clusters of microshoots (Fig. 1*A*), the number of obtained microshoots depended on the cultivar (Table 1). The highest number of microshoots was formed by cv. Hermes with an average of 13.33 microshoots per sprout, whereas only 3 microshoots per sprout were produced by cv. Spunta. These results are in agreement with Srivastava *et al*. (2012) who reported different responses of different potato cultivars to applied conditions during micropropagation.

Table 1. The responses of the initial sprouts of three potato cultivars (Agria, Hermes, and Spunta) after one month culture on MS medium supplemented with 1 mg dm^{-3} BAP and 0.5 mg dm^{-3} GA_3. Means ± SDs, $n = 3$, * indicates significant differences at $P \leq 0.05$ between Spunta and the other two cultivars.

Cultivar	Number of shoots [$sprout^{-1}$]	Length of shoots [cm]	Number of nodes [$shoot^{-1}$]
Agria	11.67 ± 1.52	4.87 ± 0.15	4.33 ± 0.57
Hermes	13.33 ± 0.57	6.23 ± 0.46	4.67 ± 0.57
Spunta	3.00 ± 1.00*	3.83 ± 0.28*	2.67 ± 0.57*

Nodal explants of all cultivars showed 100 % shoot formation but with different number of shoots per explant. The effect of the applied culture conditions was variable and depended on the genotype as it was previously reported by a number of investigators using different cultivars (Shukla *et al*. 2007, Srivastava *et al*. 2012). In general, MS medium supplemented with 1 mg dm^{-3} BAP and 0.5 mg dm^{-3} GA_3 was the best for shoot multiplication. Agria and Hermes showed higher potential for induction of shoot multiplication and growth than Spunta (Table 2). Agria was the best where several shoots were formed on medium with BAP and GA_3, and this is in agreement with Liljana *et al*. (2012) who reported that Agria cultivar has high potential for *in vitro* micropropagation and microtuberization.

All the three cultivars exhibited good rooting especially under the influence of IBA, and a concentration 1 mg dm^{-3} was the best (Table 3). These data indicated that all the cultivars were amenable to micropropagation where good rooting pattern ensures better survival percentage upon their transfer to *ex vitro* conditions. In this work, frequency of the root formation even on IBA free medium was 95 and 85 % in Agria and Hermes, respectively, but it was relatively low in Spunta (42.8 %). Analysis of variance for the measured parameters showed that the Spunta cultivar significantly differed from the Agria and Hermes cultivars, whereas it showed the lowest response to rooting induction media. Variation in root formation in dependence on genotypes was also reported by Pereira and Fortes (2003) and Yousef *et al*. (2011).

Induction of callus formation of different explant types of the three cultivars was carried out using MS medium supplemented with 0.56 mg dm^{-3} BAP, 0.11 mg dm^{-3} 2,4-D, and 0.96 mg dm^{-3} NAA. Callus mass

developed using shoot tips and nodal cuttings

Table 2. Effect of different concentrations of BAP and GA_3 on shoot multiplication of potato cultivars Agria, Hermes, and Spunta. Means ± SDs, $n = 4$, *, ** and *** indicate significant differences at $P \leq 0.05$ between MS medium supplemented with 1 mg dm^{-3} BAP + 0.5 mg dm^{-3} GA_3 and the other media types, Spunta and the other two cultivars with the same medium type, Agria and the other two cultivars with the same medium type, respectively.

Cultivar	BAP [mg dm^{-3}]	GA_3 [mg dm^{-3}]	Number of shoots [explant^{-1}]	Length of shoots [cm]	Fresh mass of shoot cluster [g]
Agria	-	1	1.66 ± 0.57*	2.95 ±0.91*	0.0358 ± 0.0009*
	1	-	2.00 ± 0.00*	2.85 ± 0.21*	0.0254 ± 0.0028*
	0.5	0.5	4.00 ± 0.00	3.63 ± 0.15	0.1520 ± 0.0675
	0.5	1	3.00 ± 0.00*	2.80 ± 0.17*	0.0228 ± 0.0009*
	0.5	2	1.00 ± 0.00*	2.20 ± 0.42*	0.0197 ± 0.0004*
	1	0.5	5.33 ± 0.57	5.26 ± 0.25	0.1631 ± 0.0012
	1	1	3.66 ± 0.57*	4.66 ± 0.15	0.6876 ± 0.0963*
	1.5	1	4.00 ± 1.00	6.00 ± 0.20	1.0111 ± 0.0010*
	2	1	5.00 ± 1.00	5.10 ± 0.36	0.6027 ± 0.0538*
Hermes	-	1	1.00 ± 0.00*	1.35 ± 0.21*	0.0153 ± 0.0015*
	1	-	1.00 ± 0.00*	1.96 ± 0.15*	0.0177 ± 0.0033*
	0.5	0.5	1.00 ± 0.00*	1.96 ± 0.25*	0.0227 ± 0.0021*
	0.5	1	1.00 ± 0.00*	2.53 ± 0.45*	0.0464 ± 0.0025
	0.5	2	1.00 ± 0.00*	2.83 ± 0.65*	0.0289 ± 0.0037*
	1	0.5	5.00 ± 1.00	5.73 ± 0.68	0.0570 ±0.0054***
	1	1	1.66 ± 0.57*	3.40 ± 0.14*	0.0756 ± 0.0055*
	1.5	1	2.00 ± 0.00*	2.63 ± 0.32*	0.0117 ± 0.0007*
	2	1	2.00 ± 0.00*	2.16 ± 0.28*	0.0552 ± 0.0021
	-	1	1.70 ± 0.14*	1.35 ± 0.21*	0.0088 ± 0.0007*
Spunta	1	-	1.00 ± 0.00*	2.00 ± 0.42*	0.0109 ± 0.0004*
	0.5	0.5	1.00 ± 0.00*	2.33 ± 0.28*	0.0088 ± 0.0002*
	0.5	1	1.66 ± 0.57*	2.56 ± 0.51*	0.0205 ± 0.0039*
	0.5	2	1.66 ± 0.57*	2.90 ± 0.14	0.0053 ± 0.0003*
	1	0.5	3.00 ± 0.00**	3.80 ± 0.20**	0.0653 ± 0.0062***
	1	1	1.33 ± 0.57*	2.35 ± 0.21*	0.0215 ± 0.0141*
	1.5	1	2.66 ± 0.57	2.55 ± 0.35*	0.0187 ± 0.0011*
	2	1	1.66 ± 0.57*	2.53 ± 0.37*	0.0185 ± 0.0033*

were significantly higher than those developed by using internodal cuttings. Generally, callus mass of any explant was significantly higher in Agria and Hermes than in Spunta (Table 4). In addition, callus medium stimulated not only callus formation but also resulted in formation of one vigour shoot from shoot tip or lateral buds of some nodal segments. Generally, 2,4-D is the most effective auxin for callus induction in potato when it is used alone or in combination with cytokinins (Khadiga *et al.* 2009, Kumar *et al*. 2010, 2014).

Healthy and vigorous microshoots were essential prerequisites to initiate microtubers with sufficient mass to resist storage and retrieval of plants. Comparison between the effect of multiplication and shoot growth medium on shoot multiplication and growth was investigated (Table 5 and Fig. 1*B,C*). Shoot multiplication medium resulted in the formation of significantly higher shoot number per explants when compared with those grown on other growth media. On the other side, shoot growth was significantly higher on shoot growth than on shoot multiplication medium in all the used cultivars. Under these conditions, Spunta showed the lowest response. Thus, growth of shoots *in vitro* depended on the genotype as it was reported by Gonzalez *et al*. (2001). Consequently, to increase shoot vigour prior microtuberization, shoots were subcultured to grow on shoot growth medium.

Under dark, MS medium supplemented with 4 mg dm^{-3} of BAP resulted in a microtuber production (Table 6, Figs. 1*D,E*). Under a 16-h photoperiod, green microtubers were obtained; it may be due to synthesis of the alkaloid solanin (Hoque 2010). Our experimental findings revealed that dark conditions were better for microtuber formation than a 16-h photoperiod; the fresh mass of microtubers formed under the dark was significantly higher than under a 16-h photoperiod. These results were in agreement with those of Sakha *et al.* (2004), who reported that microtuber formation frequency is higher under dark than light/dark conditions.

The effect of BAP in combination with KIN in comparison to BAP alone on microtuber formation was investigated (Table 7). Generally, data revealed that MS

Table 3. Root formation on microshoots of Agria, Hermes, and Spunta as influenced by different concentrations of IBA in MS basal medium for one month. Means ± SDs, n = 4, * and ** indicate significant differences at $P \leq 05$ between MS medium + 1 mg dm^{-3} IBA and other media, and Spunta and the other two cultivars, respectively.

Cultivar	IBA [mg dm^{-3}]	Responding microshoots [%]	Number of roots [microshoot^{-1}]	Length of root [cm]
Agria	0	95	3.00 ± 1.00*	4.66 ± 0.28*
	0.5	95	8.50 ± 0.70*	7.76 ± 0.11
	1	100	17.00 ± 1.00	7.02 ± 0.20
	2	100	13.66 ± 0.57*	8.23 ± 0.20
	3	100	11.66 ± 0.57*	7.20 ± 0.52
Hermes	0	85	1.00 ± 0.00*	8.20 ± 0.34
	0.5	90	3.00 ± 0.00*	7.40 ± 0.14*
	1	100	12.66 ± 0.57	9.00 ± 1.32
	2	100	12.66 ± 0.57	8.25 ± 1.06
	3	100	11.50 ± 0.70	6.30 ± 0.10*
Spunta	0	42.8	1.00 ± 0.00*	0.83 ± 0.15*
	0.5	57.1	2.00 ± 0.00*	6.40 ± 0.17
	1	100	7.00 ± 1.41**	6.75 ± 0.35
	2	100	13.33 ± 0.57*	5.23 ± 0.25
	3	100	9.50 ± 0.70*	2.53 ± 0.05*

medium supplemented with 4 mg dm^{-3} BAP + 4 mg dm^{-3} KIN was better than medium with 4 mg dm^{-3} BAP alone. It induced higher number of microtubers per shoot and/or higher percentage of microshoots formed microtubers. Several workers preferred BAP during tuberizaton (Hussey and Stacey 1984). Relatively high concentration of KIN (4 - 5 mg dm^{-3}) or BAP (4 - 10 mg dm^{-3}) in

Table 4. Effect of different explant types on callus formation in potato cvs. Agria, Hermes, and Spunta after one month incubation. Means ± SDs, n = 4**,** * and ** indicate significant differences at $P \leq 0.05$ between internode and the other two explant types, and between Spunta and the other two cultivars, respectively.

Cultivar	Explant type	Fresh mass of callus [g]
Agria	shoot tip	0.37 ± 0.0004
	node	0.31 ± 0.007
	internode	0.27 ± 0.031*
Hermes	shoot tip	0.35 ± 0.007
	node	0.37 ± 0.001
	internode	0.25 ± 0.038*
Spunta	shoot tip	0.23 ± 0.020**
	node	0.22 ± 0.012**
	internode	0.15 ± 0.003*,**

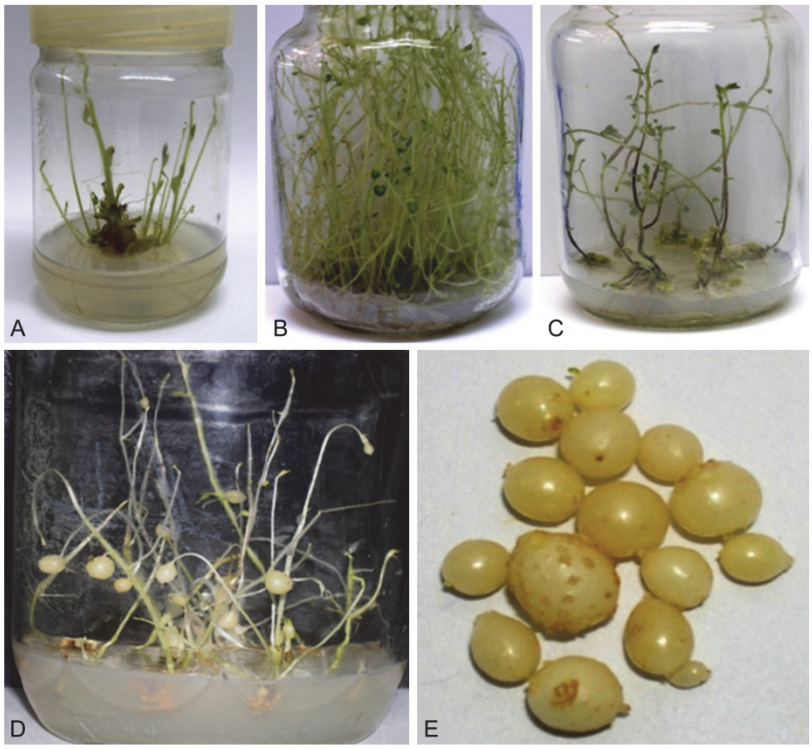

Fig. 1. Potato micropropagation and microtuber production. *A* - Shoot formation from initial sprout explant on multiplication medium for one month. Proliferation of shoot tip explant on multiplication (*B*) and growth (*C*) media for two months. *D* - microtuber formation on MS medium supplemented with 4 mg dm^{-3} BAP and 80 g dm^{-3} sucrose at 20 °C in dark for two months. *E* - harvested microtubers after two months under dark conditions.

Table 5. Effect of growth medium on the growth of shoot tip explant in comparison with multiplication medium after 40 d incubation. Means ± SDs, $n = 4$, * and ** indicate significant differences at $P \leq 0.05$ between multiplication and growth medium, and Spunta and the other two cultivars, respectively.

Medium type	Cultivar	Number. of shoots [explant^{-1}]	Length of shoot [cm]	Number of nodes [shoot^{-1}]	Fresh mass of shoot [g]
Multiplication medium	Agria	3.66 ± 0.57	7.60 ± 0.17	6.66 ± 0.57	0.035 ± 0.0002
	Hermes	5.00 ± 1.00	8.20 ± 0.36	6.66 ± 0.57	0.034 ± 0.0037
	Spunta	2.33 ± 0.57**	5.43 ± 0.25**	4.66 ± 0.57**	0.025 ± 0.0023**
Growth medium	Agria	1.00 ± 0.00*	7.63 ± 0.77	6.66 ± 0.57	0.109 ± 0.0003*
	Hermes	1.00 ± 0.00*	9.80 ± 0.26	7.00 ± 1.00	0.146 ± 0.0004*
	Spunta	1.00 ± 0.00*	6.50 ± 0.26**	5.33 ± 0.57**	0.095 ± 0.0003*

Table 6. Effect of irradiance on microtuber induction. Microshoots of cv. Hermes (about 4 cm length) were cultured on MS medium supplemented with 4 mg dm^{-3} BAP and 80 mg dm^{-3} sucrose for 2 months at 20 °C and continuous dark or 16-h photoperiod with irradiance of 100 μmol m^{-2} s^{-1}. Means ± SDs, $n = 4$, * indicates significant difference at $P \leq 0.05$ between dark and photoperiod conditions.

Conditions	Responding shoots [%]	Number of microtubers [shoot^{-1}]	Fresh mass of microtuber [g]
Dark	82.6	2.00 ± 0.00	0.0310 ± 0.0025
Photoperiod	83.3	1.00 ± 0.00	0.0261 ± 0.0017*

Table 7. Effect of lighting and cytokinins on microtuber induction. Agria, Hermes and Spunta microshoots (about 4 cm length) were cultured on MS medium supplemented with 4 mg dm^{-3} BAP or 4 mg dm^{-3} BAP + 4 mg dm^{-3} KIN, and 80 g dm^{-3} sucrose for 2 months at 20 °C. Means ± SDs, $n = 4$, *, ** fresh mass significantly different from microshoots grown on MS medium supplemented with 4 mg dm^{-3} BAP + 4 mg dm^{-3} KIN under light/dark conditions, and fresh mass of microtubers of Agria and Hermes cultivars at the same conditions at $P \leq 0.05$, respectively.

Cultivar	Conditions	Type of cytokinin	Number of cultured shoots	Frequency of microtuber induction [%]	Number of microtubers [shoot^{-1}]	Fresh mass of microtuber [g]
Agria	dark	BAP	42	80.9	1	0.0296 ± 0.0024*
		BAP + KIN	40	70	2	0.0675 ± 0.0011
	photoperiod	BAP	48	66.6	1	0.0347 ± 0.0019*
		BAP + KIN	40	90	2	0.0609 ± 0.0007
Hermes	dark	BAP	46	82.6	2	0.0310 ± 0.0025*
		BAP + KIN	40	80	1	0.0335 ± 0.0038
	photoperiod	BAP	44	54.5	1	0.0327 ± 0.0019*
		BAP + KIN	40	90	2	0.0453 ± 0.0011
Spunta	dark	BAP	48	91.6	1	0.0131 ± 0.0007*
		BAP + KIN	44	36.3	1	0.0123 ± 0.0003*
	photoperiod	BAP	44	72.7	1	0.0152 ± 0.0044*
		BAP + KIN	44	90.9	1	0.0285 ± 0.0088**

combination with high concentration of sucrose (8 %) result in efficient microtuber formation (Sakha *et al*. 2004, Hoque 2010, Liljana *et al*. 2012, Mashhadi and Moeini 2015). BAP in combination with KIN is also used (Kanwal *et al*. 2006, Diémé *et al*. 2013) to form microtubers and to improve their germination, especially when relatively high concentration of sucrose (8 - 10 %) is used (Diémé *et al*. 2013). Generally, under dark, 4 - 5 mg dm^{-3} BAP or KIN is recommended (Hoque 2010). In our work, to obtain microtubers of a sufficient mass, vigour potato microshoots were cultured for 2 weeks in 16-h photoperiod followed by 6 weeks in dark. This type of treatment was referred to as light/dark treatment (Table 7) and it was the best for microtuber production in all three potato cultivars. It increased microtuberization by enhancing tuberonic acid synthesis which plays an important role in *in vitro* tuber formation (Alisdair and Willmitzer 2001). Al-Hussaini *et al.* (2015)

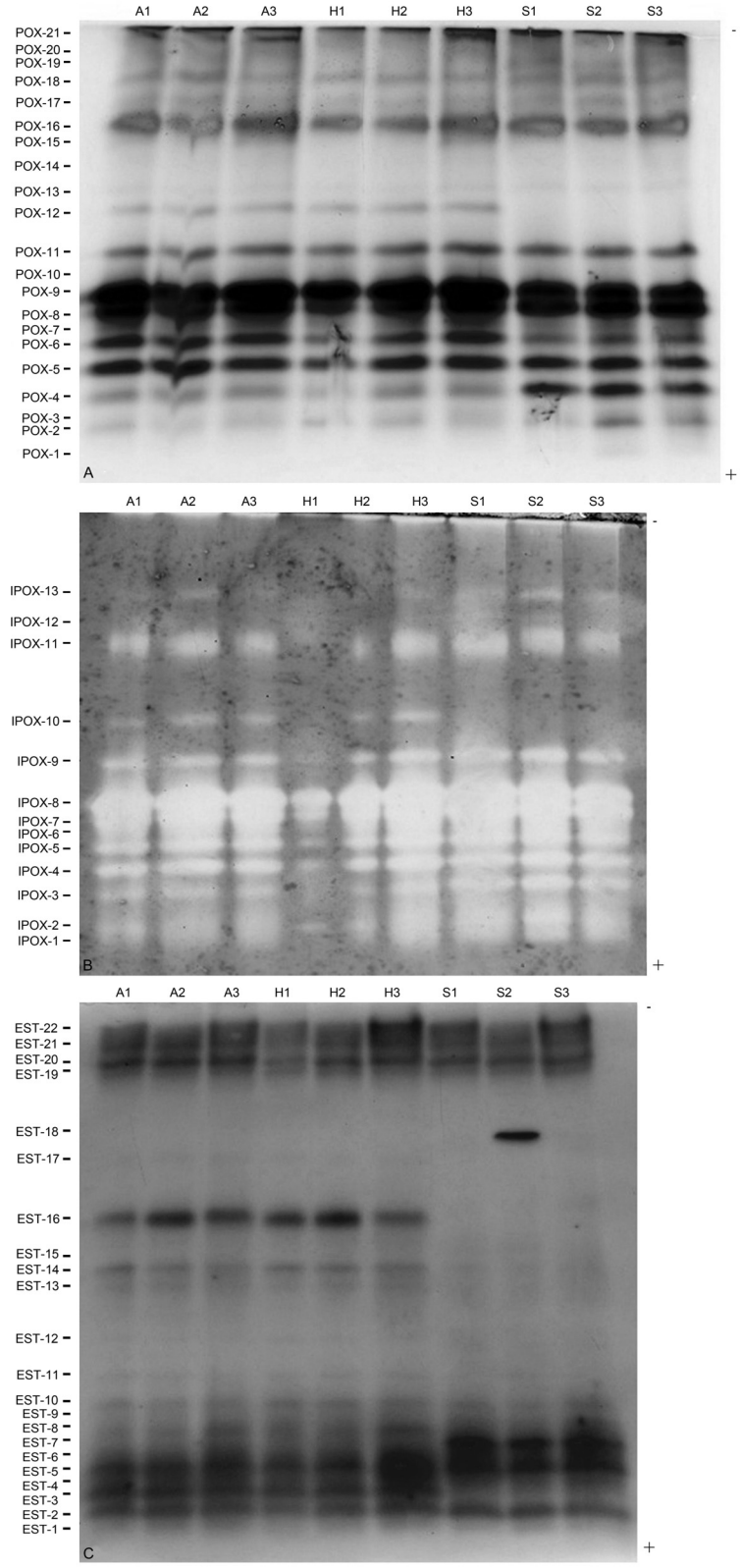

Fig. 2. Native gel electrophoresis of *A* - POX, *B* - IPOX, and *C* - EST isozyme patterns of the three potato cultivars, Agria, Hermes and Spunta (referred to as A, H, and S, respectively). Microshoots were cultured on growth medium for one month at a 16-h photoperiod, then they were transferred to multiplication, growth, or microtuber induction medium (referred to as 1, 2, and 3, respectively) and incubated in darkness at 20 °C for 15 d.

Table 8. Effect of sugar type on microtuber induction. Microshoots of three cultivars were cultured on MS basal medium supplemented with 4 mg dm^{-3} BAP + 4 mg dm^{-3} KIN + 80 g dm^{-3} respective sugar, for 2 weeks in 16-h photoperiod followed by 6 weeks in dark. Means ± SDs, n = 4, * and ** mean significant differences at $P \leq 0.05$ between microtubers produced on microtuberization medium supplemented with sucrose and other sugar types, and Spunta and the other two cultivars, respectively.

Cultivar	Sugar type	Number of cultured shoots	Responded shoots [%]	Number of microtubers [$shoot^{-1}$]	Fresh mass of microtuber [g]
Agria	sucrose	40	90	2	0.0822 ± 0.0002
	glucose	40	90	1	0.0409 ± 0.0059*
	fructose	36	72.2	1	0.0344 ± 0.0053*
	glucose + fructose	40	80	1	0.0555 ± 0.0250*
Hermes	sucrose	40	95	2	0.0963 ± 0.0021
	glucose	38	94.7	2	0.0894 ± 0.0130
	fructose	31	77.4	1	0.0652 ± 0.0197*
	glucose + fructose	40	80	1	0.0651 ± 0.0002*
Spunta	sucrose	24	87.5	1	0.0647 ± 0.0106**
	glucose	36	77.8	1	0.0202 ± 0.0032*
	fructose	26	61.5	1	0.0148 ± 0.0002*
	glucose + fructose	30	63.3	1	0.0182 ± 0.0009*

found that a 10-d irradiance before darkness improves *in vitro* microtuberization. On the other side, Sakha *et al*. (2004) reported that frequency of tuberization is the highest under dark conditions. Comparison of three cultivars indicated that the fresh masses of the microtubers of Agria and Hermes cultivars were significantly higher than of Spunta. The genotypic dependence of microtuber production is well known (Gopal *et al*. 1998, Srivastava *et al.* 2012, Mashhadi and Moeini 2015).

The highest frequency of microtuber formation was achieved when sucrose was used as carbon source in culture media followed by glucose. Fructose, on the other hand, reduced the microtuber formation frequency when it was used alone or in combination with glucose (Table 8). This indicates that incorporation of two monosaccharides (glucose and fructose) did not compensate sucrose for efficient microtuberization in potato. Khuri and Moorby (1995) reported that smaller and lower numbers of microtubers are produced on media containing monosaccharides than sucrose containing ones. The lowest number and the smallest size of microtubers formed on fructose containing media may be due to the inability of the cultured shoots to uptake fructose because it reduces the pH of the medium and elevates its osmolarity. In addition, uptake of glucose by cultured shoots may be also inhibited due to the presence of fructose (Khuri and Moorby 1995). This explains the low yield of microtubers when a combination of fructose and glucose was used as carbon source (Table 8). Sucrose at high concentration was needed to trigger microtuber formation, and high osmolarity was needed for continuous supply of the grown microtubers by starch (Khuri and Moorby 1995). The effect of sucrose on microtuber formation frequency, number, and mass of the obtained microtubers was cultivar dependent, and Agria and Hermes were better than Spunta.

Variation in peroxidase (POX) expression between Spunta and the other two cultivars (Agria and Hermes) was detected; in Spunta POX-1 was detected but POX-12 disappeared under all the tested conditions (Fig. 2*A*). In addition, staining of POX-4 band was higher in Spunta than in Agria and Hermes. Isozyme form POX-19 was detected in Agria and Spunta but disappeared in Hermes; consequently, this isozyme form could be used to distinguish Agria from Hermes. Microtuberization medium enhanced the appearance of isozymes of POX-14 and POX-21. In addition, microtuber production medium enhanced the appearance of POX-15 band in shoots of Agria and Hermes. Staining intensity of the isozyme POX-7 increased with the increase in the concentrations of sucrose and cytokinins in microtuber production medium in all three cultivars. Generally, the appearance of a new isozyme and/or increase in staining intensity of the detected bands was associated with the increase in enzymes activity as it was reported previously (Khavkin and Zabrodina 1994, Hassanein 1999). Indophenol oxidase (IPOX) expression pattern in Spunta differed from that of Agria and Hermes (Fig. 2*B*), where the IPOX-10 band disappeared and IPOX-12 appeared in Spunta only. In general, the lowest staining intensity of IPOX isozymes was detected when Hermes microshoots were cultured on multiplication medium. Studying POX and IPOX is very important because they are able to detoxify reactive oxygen species (Elstner and Osswald 1994). Consequently, they improve regeneration and proliferation of plantlets (Elstner and Osswald 1994, Poolle 1995, Hassanein 1999).

Clear variation in EST pattern between the Spunta cultivar and the other two cultivars, Agria and Hermes, under the influence of the three different conditions was detected (Fig. 2*C*). Seven isozyme forms (EST-3, EST-8, EST-11, EST-12, EST-13, EST-16, and EST-17) disappeared completely under the influence of all

treatments in Spunta and this may interpret its low multiplication rate and low production of microtubers in comparison to Agria and Hermes cultivars. On the other hand, three EST isozymes (EST-7, EST-15, and EST-18) were detected only in Spunta, and the staining intensity of EST-18 was higher when microshoots of Spunta were cultivated on shoot growth medium. This was in accordance with Biljana *et al*. (2003) who reported that the EST isozyme pattern is related to the genotype as well as to the morphogenic phase. These variations in EST expression are due to genetic differences and applications of plant growth regulators (Ibrahim *et al*. 2007).

The isozyme patterns indicated that the effect of genotypes was stronger than the effect of media types where sucrose and cytokinins concentrations were the most important. Data recorded from POX, IPOX, and EST isozyme patterns revealed that there was a close relationship between Agria and Hermes cultivars. Biljana *et al.* (2003) reported that isozymes are useful biochemical markers in potato plants.

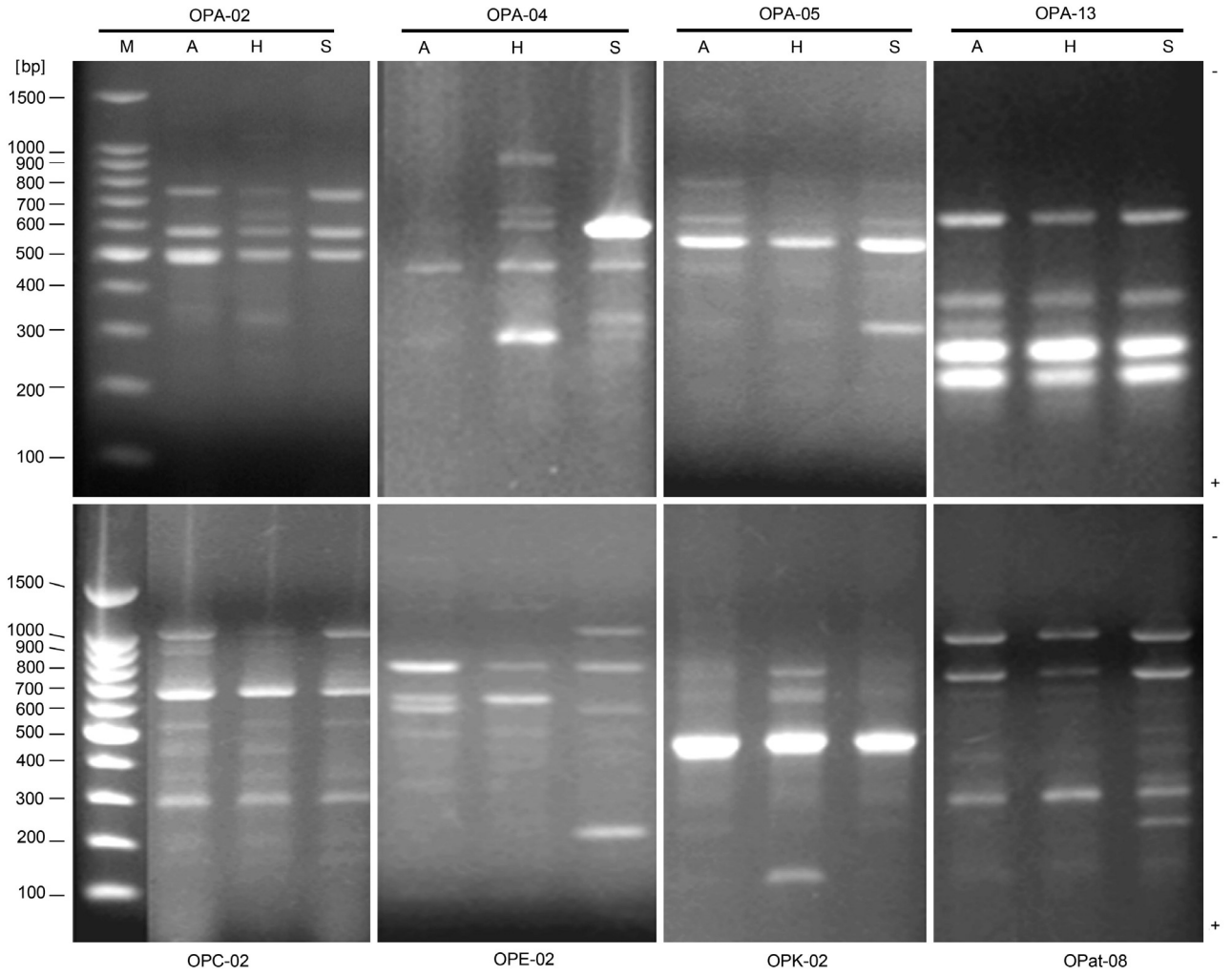

Fig. 3. RAPD-PCR profiles generated by primers OPA-02, OPA-04, OPA-05, OPA-13, OPC-02, OPE-02, OPK-02, and OPat-08. *Lane* A: Agria, *lane* H: Hermes, and *lane* S: Spunta cultivars. M: DNA ladder.

The performed RAPD profiles, using eleven arbitrary primers, detected polymorphisms between the three potato cultivars (Fig. 3). A total of 62 scorable amplified DNA fragments ranging from 250 to 2098 bp were observed. They included 33 polymorphic and 29 monomorphic bands in the three potato cultivars. The eleven RAPD primers showed a mean polymorphism of 53.23 %. The highest value (60 %) was recorded for the polymorphic primer OPA-05. Among the 33 polymorphic bands, 11 were unique markers of the three cultivars. Molecular characterization and detection of genetic variability among potato genotypes using RAPD techniques were reported in several studies (Crochemore *et al*. 2004, Rocha and Paiva 2010). The electrophoretic DNA fingerprinting indicated that Agria is closely related to Hermes. Consequently, molecular analysis explained the similarities between Agria and Hermes in response to the applied conditions. It means that the applied

conditions were favourable for micropropagation and microtuberization in Agria and Hermes but Spunta needs further studies to determine the right conditions under which it may be multiplied and formed microtuber efficiently.

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