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Assignment of Genetic Stability of Potato Microtubers cvs Diamant and Spunta through PCR Technique by Selection of Medium and Explant Types

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Abstract

This study was conducted to establish an efficient protocol for microtubers production of two potato cultivars: Diamant and Spunta by assessment of their response to different combinations of kinetin and sucrose and explant type. We also determined the genetic stability of the microtubers produced. In the first experiment, the effects of seven treatments were used. The effects of three explant types on microtuberization were compared in the second experiment. Results indicated that 0.75% Murashige and Skoog medium + 3 mgl^{-1} Kinetin + 6 or 9% sucrose using whole plantlet followed by double node were the most efficient. Moreover, genetic stability of *in vitro* potato microtubers was assessed using Random Amplified Polymorphic DNA markers. *In vitro* potato mother plantlets as a control and regenerated plantlets from microtubers acquired by the treatment 0.75% MS + 3 mg/l^{-1} Kin + 6% sucrose + 2 g/l^{-1}gelrite, explant type: Whole plantlet) were used in analysis. Polymerase chain reaction was assessed using three random oligonucleotide primers to detect genetic stability of microtubers by revealing band polymorphism. Each primer produced between 4 - 8 amplifications that ranged in size between 250 and 1100 bp. The genetic differences that detected in tissue culture-derived microtubers versus mother plantlets were low across primer used (OPA - 09 and OPB - 06). High polymorphism ratio for OPB - 05 primer. DNA analyses revealed 100% similarity among mother plantlets and its derivatives microtubers by RAPD markers while, more than 62.5 % similarity by primers OPB - 06 indicated true-to-type microtubers.

Keywords: Potato; microtuberization; kinetin; genetic stability and RAPD markers.

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1. Introduction

Potato (*Solanum tuberosum* L.) is an annual herbaceous plant, highly heterozygous tetraploid (4x = 48) belongs to nightshade family (Solanaceae) grown for its starchy edible tubers. Undoubtedly, in the world food supply and its demand have placed potato beginning in the list of food security crops recommended [1,2]. It is the world's fourth largest food crop after the three important cereals, wheat, rice and maize [3]. According to the FAO data, globally, the total area harvested in 2021, was 18,132,694 hectares produced 376 million tons of potato, the largest procedures countries were China and India, meanwhile, Egypt is in tenth rank (6,902, 816 million tons) [3]. Potato considered among the important vegetables in domestic consumption and exported abroad in Egypt where ranks the second place in the list than the other exported agricultural crops [4]. Potato is usually propagated asexually by tubers. There are two main problems associated with this conventional method; it's low multiplication rate in the field and high it's susceptibility to different phytopathogens (viral, fungal and bacterial) which cause many diseases that may be transmitted through tubers from one generation to another and affects yield and quality [5,7]. Therefore, attention has been paid to use *in vitro* micropropageted materials to overcome the problems associated production practices and to improve quality [8,9]. Plant biotechnology through means tissue culture can be used for rapid production of potato by *in vitro* multiplication with used plantlet or microtubers [10]. *In vitro* plantlets can be used for multirapid multiplication and microtuber production (*in vitro*) as well as minitubers in the greenhouse or transplant followed by cultured in the field [9].

Nowadays, microtuber production is one of the important strategies, because it has various advantages expressed as diseases free easy to handle, storage and transport where it has small size and weight, as well as identical morphological and biochemical characteristics. Moreover, it can be produced around the year [7,11,12]. Microtuberization of potato was influenced by many factors including medium composition (salts, sucrose, growth regulators and gelling agents), environmental factors, i.e., light (photoperiod, intensity and wave length) as well as temperature, genotype and explant type [11,13,14].

Many researchers reported that genotype is considered the changeable factor which cause the *in vitro* microtuberization response. Also, they added that the clone genetic origin plays the main role in *in vitro* tuberization and environmental act as stimuli agent [15,16]. Potato tuberization is a complex developmental process, it requires the interaction between genetic, environmental and biochemical factors [7,9,17,18]. Growth regulators are considered one of the most important factors affecting on *in vitro* potato initiation, multiplication and microtuberization. Supplemented MS medium with cytokinins, i.e., Kinetin has enhancing effects on different stages of *in vitro* potato microtubers' formation [8,14,19,22].

Sugars, i.e., sucrose is a crucial factor for *in vitro* potato microtuberization, as a source of energy and osmotic potent agent. It serves as a signal for the response of microtuber formation under high concentrations [7,8,13,14,20,23]. The significance of sucrose concentration for potato *in vitro* tuberization has been explained, especially when used with plant growth regulators: 20 – 30 gl^{-1} for shoot multiplication and 60 - 90 gl^{-1} for microtuber formation [8,24]. *In vitro* microtuberization process is strongly affected by explant used: Type of explant, physiological age and the density of explant. Generally, *in vitro* tuberization is induced by explant type; i.e., whole plantlet, nodal segments (single, double or more nodes) and minitubers. Explant type and size markedly affects the number and size of formed microtubers as well as development [8,10,25,26]. Taking in mind the genetic variability among potato genotypes, various methods were used to detect *in vitro* microtubers similarity. It was found that the best method for detection is molecular marker techniques [27,28]. Molecular markers are often preferred over traditional analysis. They usually detect polymorphism and genetic fidelity in several plants such as potato. A number of researchers worked on detecting potato polymorphism using molecular markers such as [29-33]. They concluded that this method was the most reliable and reproducible analysis to assess genetic stability and true to type of the *in vitro* propagated clones. The use of RAPD markers is a reliable method for detecting genetic fidelity in several plant species, including Solanum species [29,34]. RAPD markers are a quick, simple, and cost-effective method, highly discriminative, accessible and exclude the use of any radioactive probes as in restriction fragment length polymorphism. So, by using RAPD markers, we can detect true-to-type plants and enhance in vitro plants production [33]. The main goal of this investigation was to study the varietal performance of two potato cultivars: Diamant and Spunta on microtubers formation and development in relation to different combinations of kinetin and sucrose, as well as explant type. Moreover, studying the genetic stability of microtubers produced using RAPD markers.

2. Materials and Methods

The present investigation was carried out in cooperation with the plant production Dept., Fac. Technol. and Develop., Zagazig Univ. and Tissue culture Lab. of Genetics Dept., Fac. Agric., Kafrelsheikh Univ., Egypt, during the period from 2020 to 2022.

2.1. Plant material

Potato tubers (*Solanum tuberosum* L.) of two cultivars, Diamant and Spunta were obtained from Potato Brown Rot Project Unit (PBRP), Agricultural Research Center (Ministry of Agriculture and Land Reclamation). Potato sprouts of the two cultivars developed up to 1-3 cm in length were cut, rinsed with tap water for 5 min. In a laminar air-flow hood, sprout tips sterilized with 70% ethanol for thirty seconds, followed by immersion in 1% (v/v) sodium hypochlorite for 15 minutes, then rinsed four times in sterile distilled water. Sterilized shoot meristem tips (0.3 mm) were isolated under binuclear, cultured in baby jar food (five meristems/jar) containing 40 ml of basal medium contained 4.4 gl^{-1} [35], with 30 gl^{-1} sucrose + 4 gl^{-1} gelrite + 0.2 mgl^{-1} Benzyl Adenine + 0.2 mgl^{-1} Kin + 0.1 mgl^{-1}Adenine sulphate.

Meristem cultures were inoculated for eight weeks until shoots were initiated. Meristem-derived plantlets were used for further *in vitro* multiplication using single node cuttings. They were subcultured to MS medium supplemented with 30 gl^{-1} sucrose + 4 gl^{-1} gelrite + 2 mg l^{-1} BA + 2 mgl^{-1} Kin + 1 mg l^{-1}Ads to optimize shoot multiplication. Sub-culturing of single-node cuttings was done every four weeks intervals on the same shoot multiplication medium until the desired numbers of *in vitro* plantlets for subsequent experiments. The pH was adjusted to 5.8 and autoclaving at 121°C and 1.5 kg / cm^2 air pressure for 15 minutes. Culture jars were placed in a growth chamber at 25 °C ± 2 under 16 / 8 hour photoperiod (light / dark) and light intensity of 2000 Lux [36].

2.2. In vitro production of microtubers

This work included two experiments to study the effect of some factors on microtuberization of the two varieties Diamant and Spunta.

2.2.1. The first experiment

The investigation was conducted to study the response of two potato genotypes (Diamant and Spunta) to media composition. Seven media combinations were used (as shown in Fig. 2 and Table 2).

Explants (double nodes *in vitro*- cuttings) of the two cultivars (14 treatments) were cultured on 0.75% MS + 2 gl^{-1} gelrite. Cultures were incubated at 20 °C ± 2 under 8 / 16 hour (light / dark) photoperiod and light intensity 1000 Lux.

2.2.2. The second experiment

This experiment aimed to observe the varietal differences between microtubers development of Diamant and Spunta cultivars based on explant type. Different types of explants (i.e., single node, double node and whole plantlet without roots) of each cultivar were cultured on 0.75% MS

medium supplemented with 3.0 mgl^{-1} Kin and 6% sucrose with incubation under the same conditions of the first experiment.

Recorded data: (a) number of days to microtuberization, (b) microtuberization percentage, (c) number of microtubers per explant after 30 days of incubation, (d) at harvest (75 days) and (e) fresh weight of microtubers per explant (mg) at harvest.

2.3. DNA isolation and using RAPD markers to study genetic stability

Microtubers acquired by the treatment (0.75% MS + 3 mgl^{-1} Kin + 6% sucrose + 2 gl^{-1} gelrite, explant type: whole plantlet) were cultured on MS + 0.2 mgl^{-1} BA + 0.2 mgl^{-1} Kin + 0.1 mgl^{-1} Ads + 30 gl^{-1} sucrose + 4 gl^{-1} gelrite. DNA was then extracted from 50 mg leaves collected from fresh *in vitro* mother plantlets as a control and tissue cultured microtubers' plantlets (four replicates) as shown in Fig. 5. Using the Gene Direx® Plant Genomic DNA Isolation Kit (Plant) (Gene Direx, Inc. USA). DNA quality and quantity were determined using Jenway™ Genova Nano Micro - Volume Spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The isolated DNA was used for RAPD analyses.

Random amplified polymorphic DNA (RAPD) analysis of the potato genotypes was performed using random decamer primers (Eurofins Genomics, GmbH, Germany). RAPD primers used in the analysis are summarized in Table 1. The polymerase chain reaction (PCR) was performed in a total volume of 25 µl and consisted of 2 µl of DNA templates, 2 µl of $MgCl_2$, 2 µl of primer, 6.5 µl of free-nuclease H_2O and 12.5 µl of master mix (One PCR™, Gene Direx, Germany). The PCR procedure included: One cycle of 4 min at 94°C followed by 43 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C, with a final extension cycle of 8 min at 72°C. The amplified DNA products were separated using horizontal gel electrophoresis system (Bio – Rad, USA) at room temperature. The gel was run on a 1.5% agarose gel staining with ethidium bromide (0.5 µg ml^{-1}) and 1.5 gm agarose in 100 ml of 0.5X TBE buffer (Tris-borate-EDTA) at 99 V for 1 h. The bands were visualized under Axygen® Gel Documentation System – BL (Corning, USA). They were scored and compared with 100 bp DNA Ladder (H_3 RTU, Gene Direx, Germany).

Table 1. RAPD primers sequence and amplification profiles of in vitro potato cultivars

Primer Name	5` Oligo Sequence - 3`	No. of total bands	Band size range (bp)
OPB - 05	TGCGCCCTTC	6	250 - 1100
OPB - 06	TGCTCTGCCC	8	300 - 1000
OPA - 09	GGGTAACGCC	7	250 - 1500

2.4. Statistical analysis

Two experiments were conducted in Randomized Complete Design with three replicates (each replicate (jar) contained five explants). The recorded data were subjected to be analyzed by Microsoft Statistix 10 program. Means were compared using least significant difference test (LSD) at 5% level [37].

3. Results

3.1. Effect of genotype, medium constituents and their interaction

The present investigation was carried out to establish the best combination of kinetin and sucrose for production and development of *in vitro* microtubers for two potato varieties Diamant and Spunta. As for number of days for induction of microtubers, there were significant differences between the two cvs.: Diamant had shorter duration (16.47) than Spunta which took longer time (18.36). As for microtuberisation percentage, the highest percentage of microtuberisation was observed in Diamant (57.62%). Spunta showed no significant differences compared to Diamant (43.81%). The number of microtubers produced per explant was not the same for the two potato cultivars after 30 and 75 days. The number of microtubers produced by Diamant cv. was (1.72 and 2.33, respectively) compared with Spunta cv. (1.28 and 1.66, respectively). Diamant produced heavier microtubers per explant (143.86 mg), meanwhile, Spunta (131.33 mg). No significant differences were observed in this feature as shown in Fig. 1.

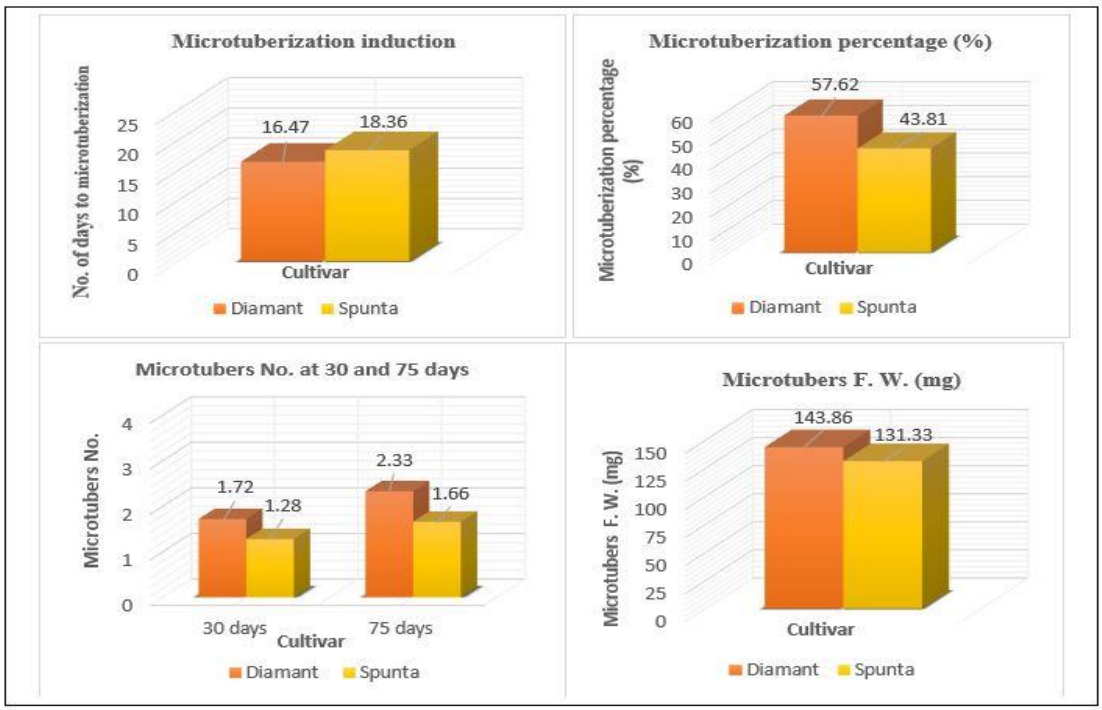
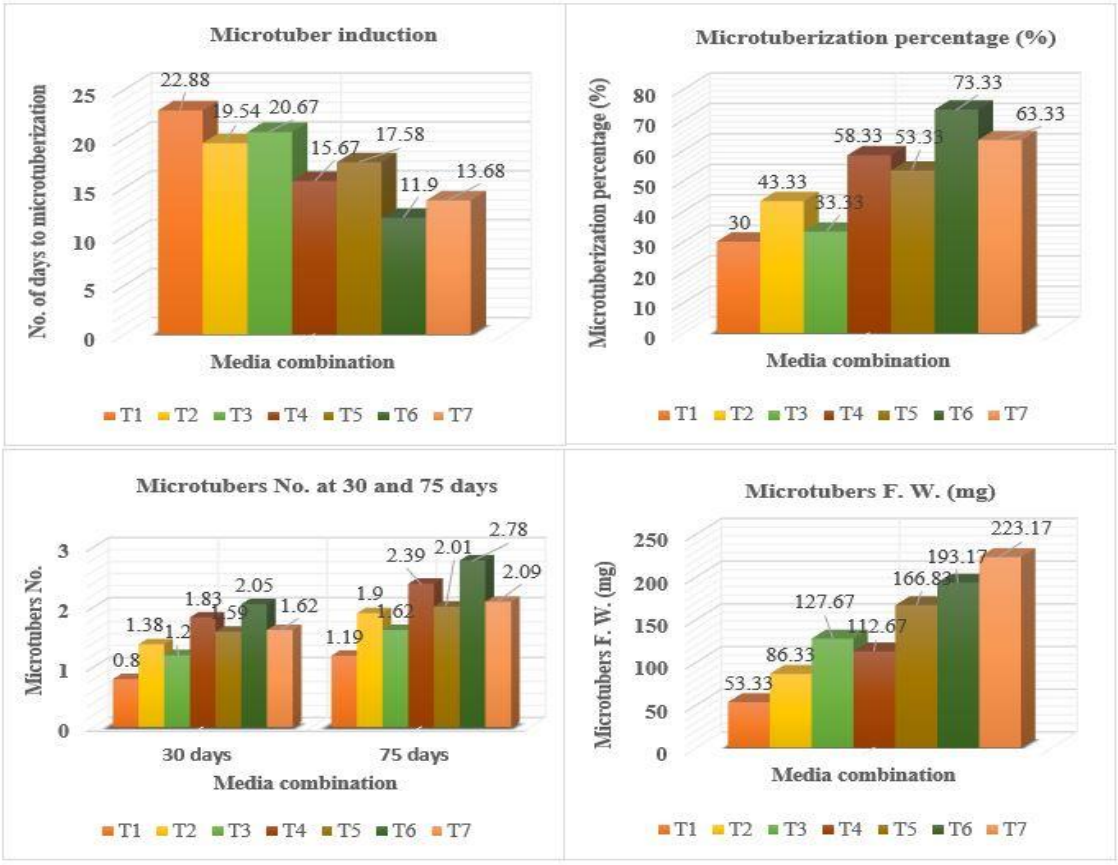


Fig.1. Effect of potato cultivars Diamant and Spunta on microtuberization.



T_1: 3% sucrose
T_2: 6% sucrose
T_3: 9% sucrose
T_4: 2mgl^{-1} Kin + 6% sucrose
T_5: 2mgl^{-1} Kin + 9% sucrose
T_6: 3mgl^{-1} Kin + 6% sucrose
T_7: 3mgl^{-1} Kin + 9% sucrose

Fig. 2 Effect of media composition on potato microtuberization

Efficiency of microtuberization is proven to be dependent on media composition, i.e., kinetin and sucrose concentrations. Days of microtubers induction significantly variated with the different concentrations of kinetin and sucrose. Maximum days of tuberization was generally observed in all treatments without Kinetin, especially in medium supplemented with 3% sucrose which took 22.88 days.

Meanwhile, supplemented MS medium with 2 or 3 mgl^{-1} Kin with 6 or 9% sucrose took the lowest period to microberize. Obtained results proved that the treatment 3 mgl^{-1} Kin + 6% sucrose was the best since it obtained the minimum days (11.9 days) for microtuberization followed by the treatment 3 mgl^{-1} Kin + 9% sucrose (13.68 days). As for the other parameters studied, application of 3 mgl^{-1} Kin + 6% sucrose resulted to the highest values of microtuberization percentage (73.33%) and number of microtubers / explant in 30 and 75 days (2.05 and 2.78, respectively), meanwhile the maximum average weight of microtubers / explant was obtained by 3 mgl^{-1} Kin + 9% sucrose. On the other hand, culture medium without Kin obtained the lowest values for all parameters studied. These results were illustrated in Fig. 2.

With regard to the interaction between genotypes and medium constituents, data in Table 2 shows that sucrose concentrations (3, 6 and 9%) without Kin gave the longest period for microtuber induction and the lowest values for all tested parameters, especially the concentration 3% sucrose for the two cultivars. Data also shows that MS medium with 2 or 3 mgl^{-1} Kin and 6 or 9% sucrose enhanced the effect of potato microtuber formation of the two varieties. Cultured explants of Diamant cv. on medium supplemented with 3 mgl^{-1} Kin + 6% sucrose was the preferred treatment which gave the least number of days for microtuberization (11.00 day) and highest values of microtuberization percentage (80.00%), number of microtubers at 30 and 75 days (2.34 and 3.23 microtubers / explant) respectively. Meanwhile, the maximum average weight of microtubers (231.00 mg) by the treatment 3 mgl^{-1} Kin + 9% sucrose. In this concern, Spunta cv. took the same trend with the treatments, but came in the second rank after Diamant cv. for the response to the applications.

Table 2. Effect of the interaction between the potato cultivars Diamant and Spunta as well as media composition on microtuberization

Treatment		No. of days to microtuberization	Microtuberization percentage (%)	No. of microtubers / explant at		Aver. of microtubers F.W. (mg)
Cultivar	Media			30 days	75 days	
Diamant	T_1	21.92 ab	33.33 ef	0.93 gh	1.35 ef	63.33 fg
	T_2	20.17 abc	53.33bcde	1.50 cdef	2.25 bcd	90.00 fg
	T_3	17.67abc	40.00 def	1.33 efg	1.83 cdef	131.67 cdef
	T_4	14.89 abc	63.33 abc	2.18 ab	2.78 ab	120.33 def
	T_5	16.61 abc	60.00 abcd	1.89 abc	2.30 bcd	171.33 abcde
	T_6	11.00 c	80.00 a	2.34 a	3.23 a	199.33 abc
	T_7	13.05 bc	73.33 ab	1.83 bcd	2.58 abc	231.00 a
Spunta	T_1	23.83 a	26.67 f	0.67 h	1.03 f	43.33 g
	T_2	18.91 abc	33.33 ef	1.25 fg	1.56 def	82.67 fg
	T_3	23.67 a	26.67 f	1.07 fgh	1.40 ef	123.67 def
	T_4	16.44 abc	53.33 bcde	149 cdef	2.00 bcde	105.00 efg
	T_5	18.54 abc	46.67 cdef	1.29 efg	1.72 def	162.33 bcde
	T_6	12.80 bc	66.67 abc	1.76 bcde	2.33 bcd	187.00 abcd
	T_7	14.30 abc	53.33 bcde	1.41 def	1.59 def	215.33 ab

values having the same alphabetical letter (s) in each column did not significantly at 0.05 level

T_1: 3% sucrose T_2: 6% sucrose T_3: 9% sucrose
T_4: 2mgl^{-1} Kin + 6% sucrose T_5: 2mgl^{-1} Kin + 9% sucrose T_6: 3mgl^{-1} Kin + 6% sucrose
T_7: 3mgl^{-1} Kin + 9% sucrose

3.2. Effect of genotype, explant type and their interaction

As for the effect of genotype, Diamant cultivar took the lowest days for microtuberization (11.77 days) and gave the greatest values for all observed parameters, i.e., microtuberization percentage (88.89%), number of microtubers / explant at 30 and 75 days (2.30 and 3.82, respectively) and the average weight of microtubers / explant (184.22 mg) compared to Spunta cv. as shown in Fig. 3. Various treatments on explant sources resulted in significant differences as shown in Fig .4. Whole plantlet gave the shortest time for tuberization induction (9.61 days), maximum tuberization percentage (90.00%) and number of microtubers / explant (2.93 and 4.94, respectively) at 30 and 75 days. Meanwhile, average weight of microtubers / explant had no significant difference between whole plantlet and double nodes as well as between single node and double node in microtuber percentage. The highest fresh weight average of microtubers (216.67 mg) was resulted by whole plantlet.

As for the interaction between genotype and explant type, data in Table 3. shows non-significant differences between whole plantlet and double nodes either with Diamant or Spunta cvs. for microtuber production compared with single node. In this concern, the culture of whole plantlet followed by double nodes of Diamant cv. gave the lowest number of days to microtuber initiation (7.99 and 11.00), tuberization percentage (100 and 86.67%), number of microtubers / explant (5.87 and 3.23) at 75 days, as well as microtuber average weight / explant (221.33 and 199.33 mg) respectively in comparison with single node. As for Spunta cv., culture of whole plantlet resulted to short period for tuberization (11.22 day), maximum number of microtubers / explant at 30 and 75 days (2.28 and 4.00 respectively) and microtubers average weight / explant (212.00 mg) compared with single and double node explants, despite there were no significant differences in most parameters between whole plantlet and double node explants.

3.3. Molecular characterization of genetic stability

The yield and quality of extracted DNA are critical for subsequent PCR analysis. The average of DNA concentration (ng / µL) is in the range of 103.63 - 917.01, the average of A260 / A280 ratio is in the range of 2.109 - 2.167 and the average of A260 / A230 ratio is in the range of 2.173 - 2.581. The averages are obtained using the selected extraction technique presented in Table 4.

A total of 21 scorable amplified bands were reproducibly obtained. Each primer produced 4 - 8 amplification products that ranged in size between approximately 250 and 1100 bp as shown in Fig. 5. and Table 5 Detected genetic differences of *in vitro* microtubers versus *in vitro* mother plants were low across used primers (OPA - 09 and OPB - 06). High polymorphism ratio was recorded for OPB - 05 primer because some microtubers did not match with this primer, so it could be excluded for both cultivars. DNA analyses revealed 100% similarity between mother plants and its microtubers-derivatives by RAPD markers, while more than 62.5% similarity was recorded by OPB - 06 primer that indicated a true-to-type microtubers.

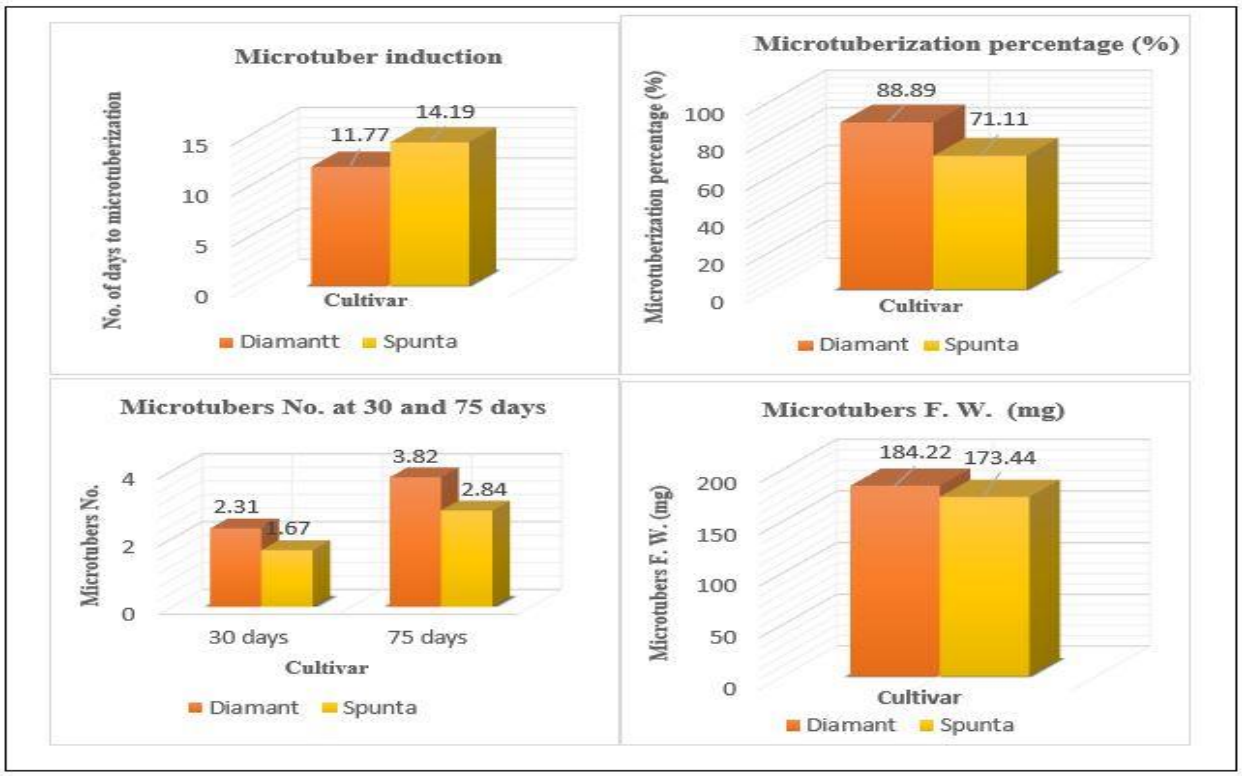


Fig.3. Effect of potato cultivars Diamant and Spunta on mcirotuberization.

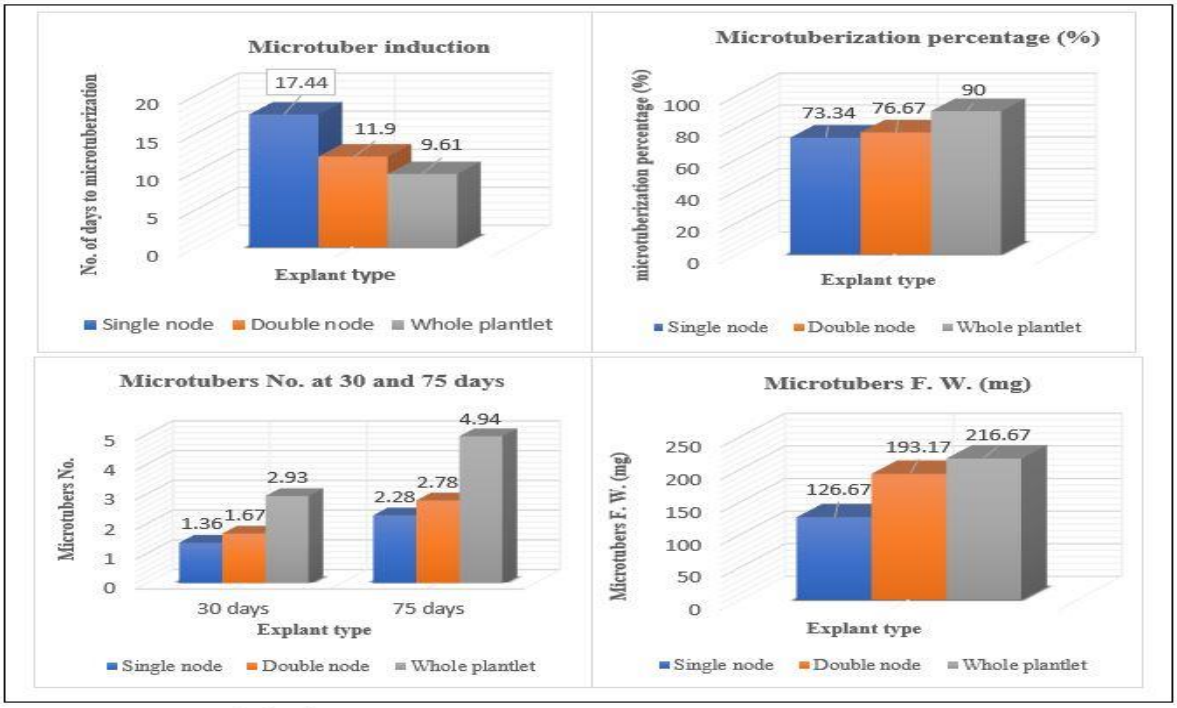


Fig.4. Effect of explant type on potato microtuberization.

Table 3. Effect of the interaction between the two potato cultivars Diamant and Spunta and explant type on microtuberization

Treatment		No. of days to microtuberization	Microtuberization percentage (%)	No. of microtubers/ explant at		Aver. of microtubers F. W. (mg)
Cultivar	Explant type			30 days	75 days	
Diamant	Single node	16.33 a	80.00 bc	1.38 cd	2.37 cd	132.00 b
	Double node	11.00 b	86.67 ab	1.94 bc	3.23 bc	199.33 a
	Whole plantlet	7.99 c	100.00 a	3.58 a	5.87 a	221.33 a
Spunta	Single node	18.55 a	66.67 c	1.33 d	2.18 d	121.33 b
	Double node	12.80 b	66.67 c	1.40 cd	2.33 d	187.00 a
	Whole plantlet	11.22 b	80.00 bc	2.28 b	4.00 b	212.00 a

values having the same alphabetical letter (s) in each column did not significantly at 0.05 level

Table 4. DNA concentration and purity using the Gene Direx Kit extraction method for all sample extracts

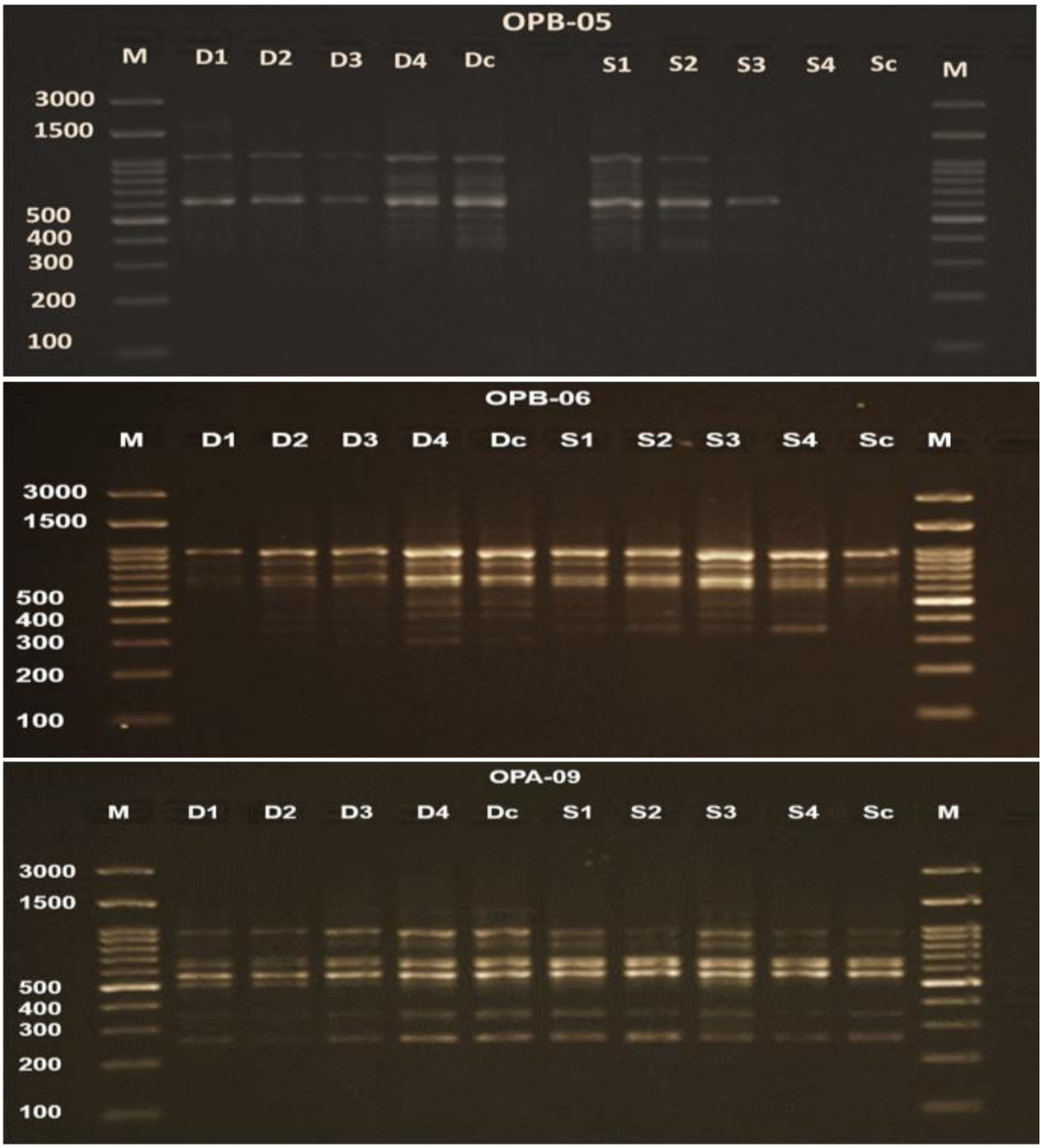
Sample	DNA Concentration (ng / µl)	A260 / A280 ratio	A260 / A230 ratio
D_C	423.27	2.140	2.255
D_1	426.42	2.155	2.247
D_2	632.47	2.109	2.223
D_3	278.85	2.120	2.195
D_4	479.53	2.126	2.173
S_C	205.67	2.151	2.581
S_1	103.63	2.112	2.331
S_2	109.69	2.153	2.265
S_3	106.55	2.143	2.357
S_4	917.01	2.167	2.202

D_C: Diamant *in vitro* mother plant D_{1-4}: Diamant tissue cultured microtubers' plantlets (four replicates).
S_C: Spunta *in vitro* mother plant S_{1-4}: Spunta tissue cultured microtubers' plantlets (four replicates).

Table 5. Total bands, monomorphic, polymorphism % by RAPD primers in two potato cultivars

Primer	Total bands		Monomorphic		Polymorphic		Polymorphism %	
	D	S	D	S	D	S	D	S
OPB – 05	6	4	2	0	4	4	66.6	100.0
OPB – 06	8	7	5	5	5	2	37.5	28.5
OPA - 09	7	7	5	7	2	0	28.5	0.0

D: Diamant **S:** Spunta



Dc: Diamant *in vitro* mother plant **D1–4:** Diamant tissue cultured microtubers' plantlets (four replicates).
Sc: Spunta *in vitro* mother plant **S1–4:** Spunta tissue cultured microtubers' plantlets (four replicates).
M= 100 bp ladder.

Fig. 5. RAPD profiles of potato genotypes generated by OPB – 05, OPB – 06 and OPA – 09 markers.

4. Discussion

There are various important factors that affects *in vitro* plantlet growth and microtuber production, i.e., genotype, media constituents (carbhydrate source and growth regulators), explant type and environmental conditions i.e., photoperiod and light intensity [7,8,11,14]. Tuber production dependence on genotype is well known. The most important factor is the genetic origin of a clone [15,16]; it plays a main role on *in vitro* potato microtuberization with interaction with environmental conditions such as photoperiod and light intensity. Each potato variety is different in its response to *in vitro* microtuber initiation and development. Diamant cultivar took a shorter period to microtuberize, produced a higher percentage of tuberization, and achieved larger values of number and weight of microtubers per explant than the other cultivar, Spunta. The variations between the two cultivars rigidly depend on genetic factors. Potato tuber production is dependent on genotype [38]. Genetic origin of clone was considered the most important factor which consequently has a main effect on *in vitro* microtuberization. Genotypic differences between cultivars in tuberization are due to gene expression behavior [9,11,12,17,18]. In this study, results showed different varietal responses of Diamant and Spunta cvs. to the various medium compositions. The medium containing 3 mgl^{-1} Kin + 6% or 9% sucrose was the best medium for tuberization and microtuber development of Diamant cultivar. Recorded results are confirmed [19,22] that stated that supplemented MS medium with kinetin had enhanced the effect of potato microtuberization. In this respect, it is known that cytokinins have a vital role in the stimulation of cell proliferation (during induction stage of tuberization) cell division, enlargement and growth [39,40]. During initiation, cytokinins, i.e., kinetin promotes tuber development; it accumulates starch activates and starch synthesis [14,41]. Kinetin acts a principal role by regulating gene expression during the transport and accumulating the assimilates towards the stolons of potato [8,42].

Sucrose is commonly used at range of 6 - 10% for microtuberization. In this respect, several studies indicated that sucrose at the concentration 6% was the best for microtuberization [8,14,23]. Moreover, there are different studies demonstrated that 8 - 10% sucrose concentration had significant effect for microtuber formation [7,13,20,24]. Sucrose is essential for tuberization as it serves as an energy source and as osmotic potential agent [5,43]. Sucrose has an effective effect on microtuberization, it causes various physiological, biochemical and morphological changes due to its conversion of sucrose to starch during the development of microtubers with increased cell division and enlargement of the stolon end followed by a higher accumulation of starch and protein [44,45]. With regard to the interaction between the two varieties and medium compositions, Diamant cv. explants cultured in MS medium supplemented with 3 mgl^{-1} Kin + 6% sucrose was the favorite treatment for all studied characteristics, i.e., No. of days to microtuber initiation, microtuberization%, No. of micotubers / explant and microtubers fresh weight. Moreover, Spunta cultivar came second in rank with the same treatment. Such results are in accordance with the results [14,19,46] which concluded that potato microtuberization is enhanced by MS medium supplemented with cytokinins, i.e., Kin with high levels of sucrose (6 - 9%).

The productivity of *in vitro* microtubers is mainly affected by explant type. The highest values for all parameters studied were achieved by Diamant cultivar with the use of whole plantlets or double nodes. Lower number of days for microtuberization and a higher number of potato microtubers per explant using cultured shoots than nodal segment [25,26,47]. They also referred that the increase in length of shoot segments disclosed to medium enhances absorption of nutrients. Moreover, several researchers reported that double node segment explant was the best for microtuber production [8,48]. On the other hand, single node explant recorded the highest percentage of tuberization [10,49]. High quality DNA is required for PCR and sequencing [50]. High quality is the most critical factor in molecular studies and miniprep kit's quality is much better than the chelex method for extracting DNA from *Astacus leptodactylus* [51].

RAPD technique could be used for detection of genetic stability of *in vitro* conserved potato microtubers. RAPD markers are one of the best markers followed by ISSRs and single locus SSR markers which used to analyze 39 potato cultivars [52]. RAPD markers technique is the ideal choice for studying and detecting the genetic variation as well as DNA fingerprinting in potato somaclone. They are handy as they can be performed easily with small amounts of DNA and can show polymorphism at high levels [53]. Genetic stability up to a level of 90 – 100% similarity in cryopreserved and non-cryopreserved potato using DNA markers [54]. However, analysis of micropropagated progenies and mother plant of lily could be grouped together in one major cluster with similarity level of 92% [55]. Which indicated that direct shoot formation from explant regeneration is a safe method for multiplication of true-to-type plants.

Genetic polymorphisms variations (15.63%) were observed among regenerants of potato derived from long-term nodal tissue culture and cell selection [34]. Genetic fidelity of *in vitro* raised plants was assessed using twenty RAPD primers, but only four primers produced amplification. DNA banding patterns of all tissue culture-raised plants and mother plants were monomorphic, indicating true-to-type planting material [33]. Based on these results, it is concluded that RAPD technique could be used for detection of genetic stability of *in vitro* conserved potato microtubers.

5. Conclusions

In vitro microtuberization experiments indicate that supplemented 0.75% MS medium with 3 mgl^{-1} kinetin + 60 gl^{-1} sucrose with whole plantlet or double node segment of Diamant cv. shows the best results for all studied parameters, while Spunta cv. comes in the second rank with the same treatment. Moreover, this work suggests that it is very important to give a special protocol for each genotype for maximized microtuber production as the response of each potato variety is unique in *in vitro* microtuberization process. Using RAPD markers, no genetic variations were detected among *in vitro* microtubers cultured on 0.75% MS medium with 3 mgl^{-1} kinetin + 60 gl^{-1} sucrose using whole plantlet explant type which is the best treatment for production of true-to-type microtubers for potato improvement.

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