Journal of New Sciences
Volume 7(2). Published July, 01, 2014
http://www.jnsciences.org

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Shoot regeneration, micropropagation and microtuberization of potato *(Solanum tuberosum* L.) cultivars

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Abstract - Alaska, Safran and Spunta are the most important varieties of potatoes used by Tunisian farmers. This study was carried out in four steps. First, study on regeneration of tissue culture protocol was studied using buds as an explant for initiation of culture in MS media supplemented with four different concentrations of an auxin : indole butyric acid (IBA). Growth proliferation showed that optimum regeneration rate was obtained with 0,5 mg/l of IBA. The regenerated plants were cultured using nodal cuttings as explants for further multiplication. *In vitro* tuberization involving a combination benzyladenine (BA) and paclobutrazol (PBZ) gave good tuberization rate. Yet, liquid media containing 5 mg/l of BA was optimal to produce microtubers for all cultivars. Microtubers were transplanted in the soil, cultured in glasshouse to produce minitubers, they produces, -7,2 healthy minitubers/plant. Microtubers cultured in medium containing sucrose (80 g/l) gave best number of minitubers/plant.

Key words: regeneration / micropropagation / microtubers / minitubers production.

1. Introduction

The potato (Solanum tuberosum L.) is of paramount importance in global as well as local nutrition in tunisian food basket., especially Alaska, Safran and Spunta varieties are planted in nearly all provinces in Tunisia (Ferjaoui et al. 2010). Potato microtubers obtained by "*in vitro*" culture from single-node cuttings are convenient for handling, storage and exchange of a healthy germplasm. "*In vitro*" microtuberization of potato constitutes the transitory phase, between "*in vitro*" multiplication and establishment of cultures in the field (Kawakami *et al.* 2004). Microtuber production represents in the same time an efficient method for obtaining a healthy material (Westerman *et al.* 2005; Uddin, 2006). In fact, they offer a lot of advantages to storage, transport and mechanization due to their little size and reduced weight. They can be planted directly in the soil and they can be produced in any period of the year. In addition, they have similar morphology and biochemical features with traditional tubers (Seabrook *et al.* 2004). The most important factors during the tuberisation period are: sugar concentration in the culture medium, nitrogen content, temperature, light conditions (Yu *et al.*, 2000, Zakaria *et al.*, 2007). Besides induction, initiation and growth of potato tubers, many interactions between "*in vitro*" conditions significantly influence the productivity and much of these interactions seem to be genetically specified (Rosu *et al.*, 2004). Nevertheless, microtubers dormancy is various, and it depends on genotype, abscisic acid (ABA), and sucrose (Ranalli, 2007). In field, the yield depend on the size of microtubers and may be optimized by producing of much more uniform and larger microtubers, by modifying the "*in vitro*" procedures (Seabrook et al., 2004; Zakaria *et al.*, 2008).

Suitability of cultivar microtuberization methods and their field performances in glasshouse or in field with comparison to plantlets is probably the most important part of microtuber utilization. Several researchs of some new methodologies to mass production of microtubers, prove that microtubers can be accepted as an alternative to plantlets. With a view of the previous reports, the involvement of paclobutrazol, benzyladenine and sucrose either used in microtuberization and minituber production media or not, seemed to be important stimulatory events of potato *in vitro* tuberization (Arregui et al. , 2003 ; Coleman et al., 2001 ; Ebadi and Iranbakhsf, 2013) The present investigation was conducted to find out the suitability of three potato cultivars to Murashige and Skoog media supplemented with different concentrations of two growth hormones and different concentrations of sucrose on microtuber and minituber performances in order to selection of suitable cultivar and media for future utilization of microtubers and minituber towards commercial seed potato program in Tunisia.

2. Materials and Methods

The research was conducted at Agronomy and *in vitro* laboratory, Department of Horticotural Sciences in the High Agronomic Institute of Chott-Mariem (Sousse, Tunisia). Main used potato varieties by tunisian agricultures: Alaska, Safran and Spunta used by were collected from main Potato Research Station.

2.1. Culture media

To study the shoot regeneration, micropropagation and micro-tuberization of potato, Murashige and Skoog (1962) medium was used. This medium contained basal salts (macro and micro) and vitamins (glycine: 2 mg/l, myo-inositol: 100 mg/l, nicotinic acid: 0.5 mg/l, pyridoxine hydrochloride: 0.5 mg/l, thiamine hydrochloride: 0.1 mg/l).

2.2. Tuber sprouting

Potato tubers were washed with water and soaked in solution of 0.3% gibberellic acid (GA_3) for 5 min. Then, they were packed in paper bags which were persevered in the dark at 21°C. Development of sprouts took 3-4 weeks. 2 to 3 mm sprouts were excised from tubers and used as explants for shoot regeneration (Figure 1).

Figure 1: Sprouted tubers after GA treatment

2.3. Shoot regeneration

The sprouts were cut into a 0,4- 0,5 cm containing one bud in each explant. The explants were washed with tap water and then rinsed in 70% ethanol. They were treated with 0,1% $HgCl_2$ (Mercury chloride) for 30 s and then washed with sterile distilled water. The explants were cultured in MS media (Murashige and Skoog, 1962), supplemented with different concentrations of indole butyric acid (IBA) (0,5, 1, and 2 mg/l) supplemented with sucrose (30 g/l) and agar (8 g/l). The pH was adjusted to 5,8 before autoclaving the medium. The cultures were incubated at 25 + 2°C under 16 h light periods. Apical buds evolved into plantlets, in callus, or necrotic.

2.4. Culture initiation

Nodal segments from regenerated plantlets were used as explants for culture initiation in a MS solid medium supplemented with sucrose (30 g/l) and devoid of hormones. Cultures were maintained at 25 + 2°C. Subculture was done each 4 weeks. Later, micropropagated plantlets tested virus-free using enzyme linked immunosorbent assay (ELISA) were used for *in vitro* tuberization tests. The use of the ELISA for the detection of plant viruses use the double antibody sandwich method. This method proved to be very valuable detection tool for plant viruses (Lommel *et al*. 1982).

2.5. In vitro tuberization

After shoot proliferation, 7 plantlets were inoculated in each 250 ml glass jars containing 50 ml of tuberization media. For tuberization, combination of three concentrations of BAP (0 ; 5 and 1 mg/l) and paclobutrazol (PBZ) (0 ; 0,25 and 0,5 mg/l) were used with ½ strength MS liquid medium supplemented with 80 g/l of sucrose.

Tuberization media were tested in three different consistency: solid (agar : 8 g/l), semi-solid (liquid microtuberization media added to a solid micropropagation media) and liquid (agar free).

The plantlets from all tuberization media were kept at 25 ± 2°C temperature under complete darkness for the duration of 5 weeks. For each combination of hormones and for each concentration of sucrose 10 jars were prepared, each jar contains 7 plantlets. The percentage of tuberized plantlets was measured.

2.6. Microtuber culture

Microtubers were inoculated in culture media containing half strength (MS basal media) supplemented with different levels of sucrose (0; 40; 80 ; 120 and 160 g/l)). Cultures were incubated at 18 to 20°C in dark room/condition. The number of microtubers / plant, fresh dry weights of microtubers, and diameter of microtuber were determined.

2.7. Minituber production

The plantlets having 5-6 nodes with leaf, root mass were transferred to bacs containing mixture of sand: vermin: compost: cocopit in ratio of 1:1:1 v/v and drenched with fungicide (Bavistin) under glasshouse. Three to four mist irrigation was given to keep soil moist and to maintain the humidity for initial one week.

2.8. Experimental design and statistical analysis

In vitro and *in vivo* experiments were conducted using Completely Randomized Design (CRD). The analysis of variance was performed using SAS software through the General Linear Model (Proc GLM) and the Student Newman Keuls (SNK) test for

comparison of means. For culture initiation, 12 explants were tested for each concentration of IBA, for in vitro tuberization 7 plantlets were tested in each glass jar, and 10 jars were tested for each treatment (BA+PBZ) and for each consistency of media. For microtuber culture, 60 microtubers were evaluated for each concentration of sucrose. For minituber production, 30 minitubers were tested for each concentration of sucrose.

3. Results

3.1. Shoot regeneration

Observation of shoot regeneration was recorded from 48 explants. Results in Table 1 indicate that Shoot and root formation were found to be better with treatment of IBA (0,5 mg/l) for all cultivars. The percentage of apical buds proliferated in hole plants tubers was higher in Spunta (52%) and negligible in Safran (26%). tuberization.

Table 1 : Percentage of apical buds proliferated in plantlets, proliferated in callus or necrosis

Cultivars	**Alaska**				**Safran**				**Spunta**			
Concentrations IBA (mg.l^{-1})	**0**	**0.5**	**1**	**2**	**0**	**0.5**	**1**	**2**	**0**	**0.5**	**1**	**2**
Apical Buds Proliferated in Hole Plants (%)	0	39±_	0	0	0	26±5	0	0	0	52±11	4±1	0
Apical Buds Proliferated in Callus (%)	0	17±3	26±4	22± 3	0	41±8	10±3	8± 2	0	12±3	10± 3	14±5
Aical Buds Necrosis (%)	0	44±7	74±11	78±12	0	33±6	90±15	92±11	0	36± 8	86±14	86±11

Regenerated plants (Figure 2) were sub-cultured by micropropagation for further multiplication using nodal cuttings each four weeks. Micropropagated plants (having 5-6 nodes with leaf and sufficient amount of root mass were used for *in vitro* tuberization).

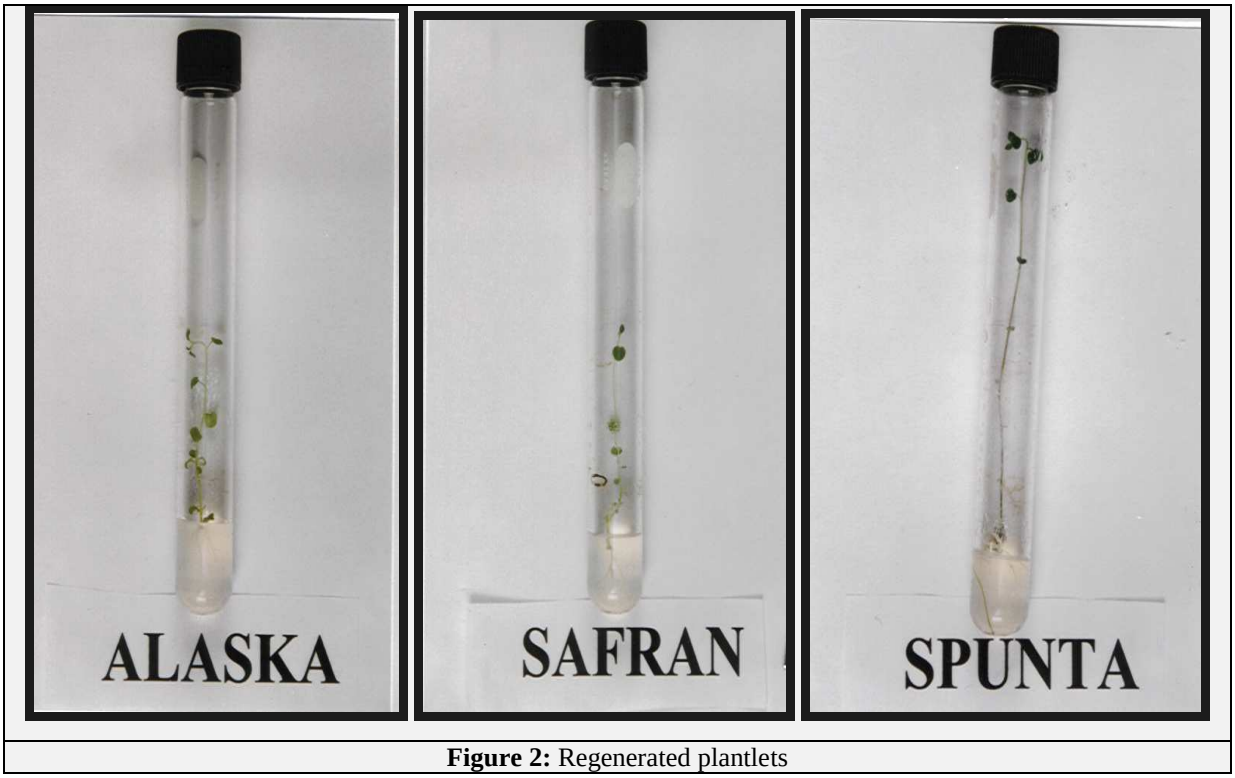

Figure 2: Regenerated plantlets

3.2. In vitro tuberization

The perusal of data in Table 2 indicates that microtuberization was not obtained without growth regulators. Otherwise, higher percentage of microtuberization was obtained in Spunta cultivar for almost all treatment. For this cultivar percentage of microtuberisation was maximum in liquid media (76%) (T4:

BA= 5 mg/l) (Figure 3), followed by semi-solid media (67%) (T5: BA= 5 mg/l+ PBZ = 0.25 g/l) and was lowest in solid media (54%) (T3: PBZ = 0.5 mg/l). Thereafter, tuberized plants in liquid media were cultured in different concentrations of sucrose.

Table 2: Percentage of tuberization in different media with different treatments

Media	Solid media			Semi-solid media			Liquid media		
Treatments (mg)	Alaska	Safran	Spunta	Alaska	Safran	Spunta	Alaska	Safran	Spunta
T1: BA(0)+ PBZ (0)	0	0	0	0	0	0	0	0	0
T1: BA(0)+ PBZ (0.25)	12±3	11±3	44±13	17±7	8±3	44±12	21±5	25±6	9±5
T1: BA(0)+ PBZ (0.5)	22± 4	14±5	54±11	14±3	18±7	32±6	27±11	26 ±7	13±5
T1: BA(5)+ PBZ (0)	13±7	16±3	31±10	11±5	11±6	29±9	33±10	21± 5	76±(18)
T1: BA(5)+ PBZ (0.25)	19± 4	13±6	27±11	15±6	17±3	67±18	45±11	17±4	58± 13
T1: BA(5)+ PBZ (0.5)	12± 3	15±8	36±8	28±7	9±5	61±13	19±8	18±3	68±21
T1: BA(10)+ PBZ (0)	14±2	6±2	3±0.9	13±4	9±1.8	23± 8	28±7	12± 2	42±19
T1: BA(10)+ PBZ (0.25)	8±2	3± 0.7	7±2	8±2	8±2	27± 6	17±2	6±2	31±7
T1: BA(10)+ PBZ (0.5)	1±0.4	2±0.9	11±3	5± 4	7± 4	18± 5	11±4	5±4	24±11

Figure 3: Tuberized plants in liquid media (Spunta cultivar)

3.3. Microtuber culture

Microtuberization of potato cultivars i.e., Alaska, Safran and Spunta affected by sucrose concentrations are illustrated in Table 3. Data indicate that cultivar Spunta produced significantly higher number of microtubers (13,83), followed by Safran (6,4) and by Alaska (3,7). In addition it produces more large size microtubers (1.74 cm) (Figure 4) which was followed by Safran (1.22 cm), whereas, it was lowest in Alaska (1.19 cm).

Table 3: Microtuberization of potato cultivars i.e., Alaska, Safran and Spunta on medium containing different concentrations of sucrose

	No. of microtubers		
Sucrose (g /l)	Alaska	Safran	Spunta
40	2.1±0.7	2.5±0.8	4.40±1.6
80	3.7±1.1	6.4±2.1	13.83±3.8
120	1.9±0.6	2.9±0.9	4.56±1.5
160	3.5±1.3	2.9±1.3	6.00±2.2

Figure 4: Microtubers (Cultivar Spunta) cultured in liquid media (Sucrose : 80 g/l)

Results presented in Table 4 reveal that average fresh and dry weight of large size microtubers was significantly higher in Spunta cultivar (73,53 and 7,47 mg respectively). So we notice that after four weeks of culture, morphological parameters of microtubers varied significantly among cultivars, yet medium supplemented with 80 g/l gave best results for all cultivars and for all parameters.

Table 4: Microtuberization of potato cultivars i.e., Alaska, Safran and Spunta on medium containing different concentrations of sucrose

Sucrose ($g\ l^{-1}$)	Fresh weight (mg)			Dry weight (mg)			Diameter (cm)		
	Alaska	Safran	Spunta	Alaska	Safran	Spunta	Alaska	Safran	Spunta
40	46.77±9.23	45.21±9.12	73.53±7.86	5.11±0.71	4.19±0.69	7.47±1.16	1.12±0.42	1.57±0.03	1.47±0.06
80	63.43±8.56	59.86±11.12	103.73±13.19	7.18±0.85	6.31±0.43	14.43±2.3	1.19±0.69	1.22±0.04	1.74±0.07
120	44.10±7.63	27.53±6.33	50.33±8.68	5.21±0.71	1.93±0.38	7.17±1.56	0.79±0.19	0.89±0.02	1.12±0.05
160	21.87±5.16	31.54±7.18	53.60±7.94	2.76±0.34	2.98±0.52	7.61±1.79	0.91±0.21	1.11±0.05	1.4±0.07

3.4. Minituber production

Although number of minitubers vary significantly among cultivars and among different concentrations of sucrose, it is raised from 4,1 (Safran, sucrose: 160 g/l) (Table 5) to 7.2 (Spunta, sucrose : 80 g/l) (Figure 5). While with the increasing concentration of sucrose inhibit the average number of minitubers. Cultivar Spunta gave highest minitubers whtaever the concentration of sucrose was.

Figure 5: Minitubers production in glasshouse

Table 5: Minituber production of potato cultivars i.e., Alaska, Safran and Spunta on medium containing different concentrations of sucrose

Sucrose	Number of minitubers/planted microtuber		
	Alaska	Safran	Spunta
40	5.3±1.2	5.2±0.7	5.5±0.8
80	5.5±1.1	5.8±1.1	7.2±1.5
120	4.7±0.9	4.9±0.8	5.3±1.3
160	4.2±0.8	4.1±0.7	6.4±1.2

4. Discussion

The analysis of regenerated plants revealed that *in vitro* growth of potato plants were affected by the concentration of IBA, it also varied significantly among the cultivars. In fact, the concentration of 0.5 mg/l IBA gave the best regeneration rate (52%). This concentration is also recommended for the regeneration of apical buds of other bulb species such as gladiolus (Bettaieb *et al.* 2007). Otherwise, some of the apical buds evolve into callus or gave necrotic roots. This necrosis is a physiological phenomenon that might be due to the depletion of the culture medium nutrients. It may be due to oxidation of polyphenolic compounds secreted by the cells (Le and Thomas 2009), or due to the production of ethylene by the callus cells, which causes cell death. Similar phenomenon was reported in the *in vitro* culture of the carob tree (Gharnit and Ennabili 2011).

The cultivar Spunta gave the best response of all the essential characters of regeneration, micropropagation and microtuberization such: percentage of regeneration, percentage of tuberization, number of microtubers/plant, fresh, dry weight of the microtuber as well as diameter of the microtuber. Variable response of different cultivars under similar *in vitro* conditions indicates that *in vitro* response of potato plant is governed by genotype. Genetically controlled response of potato, for various *in vitro* quantitative characters have been reported by earlier workers (Donnelly *et* al. 2003). Gopal *et al.* (2004) have also reported significant response of potato genotypes on the production of microtubers. Variability production of microtubers has also been reported by earlier workers (Badoni and Chauhan, 2009). Low heritability and genetic advance for microtuber production makes this trait more amenable to improvement through nutrition or cultural manipulation *in vitro*). Other workers suggested that he cultivar producing more number of microtubers per flask, higher percentage of large size microtubers and combined with high fresh weight would be best suited for microtuber based seed potato production (Kamarainen-Karpinnen *et al.*, 2010).These findings confirms that cultivar Spunta is suitable for microtuber production.

On the other hand, we noted that microtubers productions varied depending on culture medium solidification: the best tuberization was obtained in liquid media. This can be explained by that the agar present in the solid and semi solid media can impede tuberization as it contains alginic acid and D-mannitol that might chelating minerals and prevent their absorption, which prevents the accumulation of starch and consequently tuber formation (Kamarainen-Karpinnen *et al.*, 2010; Mercier *et al.*, 2011). Concerning sugar, it's presence in the culture medium is required for *in vitro* tuberization of potato. Its hydrolysis allows the synthesis and the accumulation of starch and patatin via cytoplasmic enzymes involved in sucrose metabolism (sucrose phosphate synthase (SPS), sucrose synthase (SuSy) and Invertase) (Du Jardin *et al.*, 1995; Roitsch and Ehness, 2000; Kanwal *et al.*, 2006). Although, the presence of sugar is required for tuberization, it can be a limiting factor at certain concentrations. Indeed, the variation in sucrose concentrations (40 to 160 g/l) shows that 80 g/l of sucrose had potentiality to produce heavier microtubers (microtuber weighing 104 mg, 14 microtubers / plants). While low concentrations (40 and 60 g/l) are not sufficient to allow good tuberization. Therefore, the levels of glucose and fructose obtained from the sucrose are insufficient to stimulate hexokinases, involved in the phosphorylation of these two hexoses (Farrar *et al.*, 2000; Iraqi *et al.*, 2004), so tuberization is reduced. Furthermore, high concentrations (120 and 160 g/) limit also tuberization (reduced fresh weight of microtubers and reduced number of microtubers / plant) which is due probably to an inhibition activity of

sucrose synthase (SuSy) (Roitsh and Gonzalez, 2004; Chichinska *et al.*, 2008). The present finding is a proof of the previous workers. In fact, El-Sawy *et al.* (2007) and Chichinska *et al.*, (2008) studied the influence of several factors affecting *in vitro* tuberization in potato. They reveal that the highest tuberization was achieved when 80 g/l of sucrose was added to culture media. They also indicate that higher concentrations of sucrose cause a decrease of SuSy activities, and reduce consequently tuberization.

These results are supported by the findings of Uddin (2006), which showed that the presence of sucrose (80 g/l) was beneficial and led to the production of slightly larger microtuber and higher yield. Similarly, fresh and dry weight of microtuber as well as the diameter of the microtubers were found superior at sugar concentration of 80 g/l, which was also reported by Fatima *et al.*, (2005). And significantly, slower microtuber growth rates were observed when sugar concentration was 40 g/l instead of 80 g/l; this is in line with studies of Yu *et al.* (2000). From the present investigation it can be concluded that low level (40 g/l) or too high levels of sucrose (120 and 160 g/l) was not found suitable for the microtuber production under *in vitro* conditions. Similar results were also supported by El-Sawy *et al.* (2007) and Hoque, (2010).

Although minituber production was important in Spunta cultivar produced, minituber production varies slightly among cultivars. We suppose that *in vivo* minituber production potential of potato cultivars did not have any relation with in *in vitro* microtuber production potential. Such hypothesis is supported by El Nagar and Mekawi (2012), they reported that *in vitro* yield performance of potao is not an accurate measure of field performance. Otherwise other authors have reported that tuber yield and related characteristics can be evaluated *in vitro* and will reflect *in vivo* (Ranalli *et al.* 1994; Naik *et al.* 1998). In the same whay they indicate that *in vitro* growth rate and subsequent productivity of minitubers has been reported to be genotype specific.

5. Conclusion

In the present study, the effect of two growth regulators: BA and PBZ and sucrose on performance of microtubers and minitubers in three potato cultivars: Alaska, Safran and Spunta was evaluated. Microtubers were produced from in vitro grown plantlets regenerated in MS medium supplemented with IBA (0.5 mg/l). Best microtuberization rate was obtained in MS/2 liquid media supplemented with sucrose (80 g/l) in Spunta cultivar. The highest number of microtubers (13 microtubers /plant) was obtained also in Spunta cultivar. It gaves in addition maximum fresh weight (103 mg) and larger (1.74 cm) microtubers. In glasshouse, minituber growth was better with microtubers obtained with 80 g/l of sucrose (7.2 minituber/plant). Production of large microtubers is important for successful utilization of microtubers in seed potato production (Jin et al., 2013; Rahman et al., 2013; Sonnewald and Sonnewald, 2014). Hence, this investigation propose an economical and reproducible method to obtain larger microtubers under laboratory conditions and it could be experimented to produce seed potato from in vitro grow tubers.

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