In vitro Protoplast Isolation and Regeneration of *Solanum tuberosum* cv. Binella and Burren

***Al-Maarri K.*[1]✉, *Al-Qabbani N.* [2] ,*Al-Biski F.*[2]**

ABSTRACT

The present study aims to develop simple reliable method for protoplast isolation, culture and regeneration from the leaves of potato cvs. Binella and Burren. Optimal conditions for protoplast culture, callus initiation and regeneration were determined. Moreover, genetic diversity between regenerated and mother plants, was evaluated by Inter Simple Sequence Repeats (ISSR) technique. Protoplasts were isolated from in vitro explants after four weeks multiplication medium containing 50 µM of Silver Thiousulfate (STS). Protoplast division was started after 4 days in the cv. Binella, and after 5 days in the cv. Burren, on medium B containing (Murashig and Skoog, 1962) with 1 or 1.5 mg/L of Zeatin (Z). Maximum rate of cell colonies was recorded when 1.0 mg/L Z was used, with significant differences between the two cvs. Binella and Burren (1.58 ± 0.1%, 1.27 ± 0.09%), respectively. Maximum percentage of shoot regenerated from Potato callus came out, on medium with of 0.1 mg/L naphthalene acetic acid (NAA), with significant differences between cvs. Binella and Burren (51.12 ± 8.77%, 18.96 ± 2.81%), respectively. Maximum shoots regenerated from cv. Binella callus was (50±17.68%), on regeneration medium containing 1.5 mg/L of Z, while in cv. Burren callus did not form shoots. Two months later 92% of plantlets were successfully acclimatized, in green house. Average plant length reached 26.6 cm. Also, tubers were produced with a diameter mean of 13.17 mm and average weight of 2.06g. ISSR technique showed that regenerated plants from cv. Binella callus, were identical to the mother plants by 100%.

Keywords: Potato, Manitol, True to Type, ISSR, Silver, Thiosulfate, Zeatin.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the fifth important crop that grown world wide because of it is economical (Zuba and Binding, 1989). Potato tubers are used as a source of starch, and proteins, rich in some essential amino acids (Ross, 1986). In Syria, potato total harvested area reached about 34855 hectares, which produced more than 700 000 tons in 2009 (Annual Statistical Abstract issued by the Central Bureau of Statistics in Damascus, 2009). Classical breeding of potato is difficult because of its vegetative propagation. A new cultivar can be obtained only in 8-10 years after a thought full selection of a great number of plants (Mackay, 1987). On the other hand, new strategies of plant genetic manipulation are to be considered as an important scheme. Therefore, important characters, like: resistance to viruses (*S. Tuberosum* + *S. brevidens*) could be possible through somatic hybridization with wild *Solanum* (Pehu *et al*., 1990). Moreover, other character such as resistance to *Phytophtora infestans* and *Globodera pallid* (*S. Tuberosum* + *S. circaeifolium*) (Mattheij *et al*., 1992) through somatic embryogenesis could be used.

Development of protoplast systems in plant tissue

[1] Faculty of Agriculture, Damascus University, Damascus, Syria.
[2] National Commission for Biotechnology, Damascus, Syria.
✉ kmaarri@yahoo.com
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culture technique has increased the versatility of plants for use in both biochemical and genetic research (Khatun and Flowers, 1995). This is because the cell surface membrane of the isolated protoplast is fully exposed and accessible. Thus, it would provide the starting point for genetic modification of plant cells and whole plant (Ling *et al*., 2009) which were found to be recalcitrant to *in vitro* regeneration (Zhu *et al*., 2005). Successful plant regeneration has now been achieved for a wide range of cultivars and breeding lines in potato. These include Russet Burbank, Katahdin, Kennebec, Maris Piper, King Edward and Majestic (Haberlachet al., 1985; Foulger and Jones, 1986). In addition, plants have been regenerated from dihaploid potatoes and several other *Solanum* species (Binding *et al*., 1978). Potato protoplasts have been cultured successfully for many tetraploid or dihaploid lines and for more than 30 wild species (Ferreira and Zelcer, 1989).

Protoplast fusion has been used successfully for the transfer of wild tolerance into cultivated potato, such as the resistance to viruses (Valkonen and Rokka, 1998) and frost (Preiszner *et al*., 1991) from *Solanum brevidens*, and the resistance to bacterial wilt from *Solanum commer-sonii* (Laferriere *et al*., 1999). Many cultivars of wild potato spesies has tolerance for disease, but are not compatible sexually with commercial potato cultivars, but it can transfer many of the resistance from the wild species to commercial by protoplast fusion (Laurial 2004).

Protoplasts are usually produced by treating mesophyll tissue with amixture of cell wall degrading enzymes in solutions which contain osmotic stabilizers to sustain the structure and function of protoplasts (Chabane *et al*., 2007). The most important factors for potato protoplasts liberation from leaves are the physiological state of plant material, the kinds of degrading enzymes, the composition of the reaction solution and the concentration and type of osmotic stabilizers (Davey *et al*., 2005).

ISSR markers have used in genetic studies of the potato to be effective in detecting very low levels of disparities genetic (Klliker *et al*., 2003), and successfully applied in studies of genetic diversity in a large number of plants, including potatoes (*Ellouza et al*., 2006). An efficient methods for protoplast isolation and regeneration have to be developed, as a first step for potato genetic manipulation through inter- or intra specific somatic hybridization. Therefore, the aims of these investigations were protoplast isolation and regeneration from *in vitro* leaves of tetraploid potato cvs. Binella and Burren.

MATERIALS AND METHODS

Establishment and Maintenance of Shoot Cultures

Two potato cultivars"Binella" and"Burren" (class Elite) were used in this study**.** Tubers culture were washed under running water, and then treated with GA_3 (4 ppm) for 15 min. Tubers were excised and cultured in pots containing peat-moss, under green house condition. Rose-ends were treated with alcohol 70% for 60 sec then, immerged in sodium hypochlorite 0.5 % for 10 min, and washed for 3 times with sterilized distillated water for 5 min each time. Shoot tips (0.5-1 cm) in length were removed and cultured in aseptic conditions on initial culture medium (Table 2- A). Then cultured explants were incubated in a growth room, under a temperature regime of 22 ± 2°C and photoperiod cycle of 16/8 h as light/dark, provided by fluorescent tubes with light intensity of 30 $\mu Mm^{-2}S^{-1}$ (Jayasree *et al*., 2001). Shoots obtained from the initial culture, were divided and subcultured several times on the same medium (Table 2-A), in order to obtain enough plant material for protoplasts isolation.

Protoplast Isolation

Microshoots (10 to 20 plantlets from each cultivar) were taken, cut to microcuttings 1.5 cm in length and

transferred to hormone free medium (Schenk and Hildebrand, 1972) supplemented with 50 µM of Silver thiosulfate (STS) for 4 weeks. Then explants were kept at 10 °C in darkness for 24 hours for one day before the protoplast isolation. Approximately 1.0 g of large leaves were removed, cut into thin strips (0.5-1.0 mm) with a sterile scalpel, and were plasmolysed (Table 1) (Albiski, 1995).Protoplasts were released by incubated petri dishes overnight in dark 14 h on medium contining enzyme solution (Table 1). Then, digested plant tissue was investigated under microscope. The protoplast suspension was immersed in enzyme solutionand passed through 8 layers of sheets into a fresh petri dish. The sheets were rinsed with a sterile solution of W5 (Table 1) (Menczel *et al*., 1981). Protoplast suspension derived this way was divided into centrifugation tubes (15 ml) and centrifuged at 700 rpm for five min. The supernatant was carefully removed with a Pasteur pipette, then 5.0 ml of W5 were added and the pellet was re-suspended. Centrifuge again at 500 rpm for 6 min. Protoplasts were resuspended and washed twice in wash solution (Table 1). Protoplasts density was determined using haemocytometer (Invitrogen 2002), and the viability of protoplasts was estimated by Evans blue dye 0.4% (w/v) (Ling *et al*., 2010).

Table 1. Medium composition used for isolation protoplast from potato leaves.

Plasmolysed solution		**Enzyme solution**		**Washing solution (W5)**	
Compound	**Concentration (mg/L)**	**Compound**	**Concentration (mg/L)**	**Compound**	**Concentration (mg/L)**
$CaCl_2.2H_2O$	147	Cellulase TC	%1.75	NaCl	8775
Manitol	91070	Pectinase	%0.4	KCl	745
MES	585750	$CaCl_2.2H_2O$	735	$CaCl_2.2H_2O$	18375
pH	5.6	Manitol	91070	Glycine	0.75×10^{-3}
		MES	1952.4	Glucose	0.99×10^{-3}
		pH	5.6	pH	5.8

Protoplast Culture

Freshly isolated protoplasts were suspended in the culture medium (Table 2-B) (Kikuta *et al*., 1984) at a density of 2×10^4 protoplasts/mL. 2.0 ml of protoplast suspension was plated into plastic petridishes (60 mm). The petri dishes were sealed with parafilm and incubated at 25 °C in the dark**.** The culture media were supplemented with different concentrations of Zeatin (0-0.5-1.0-1.5) mg/L. After 2 weeks, the protoplast cultures were diluted with 1.0 ml of low osmotic (0.3-0.4 M) fresh liquid culture media

Callus InitiationStage

Protoplasts colonies were transferred to callus initiation medium (Table 2-C) for callus induction. Her by, the effect of different concentrations of NAA (0-0.1-0.25-0.5) mg/L on callus induction and development were investigated. One month later, the frequency of calli derived from protoplasts was evaluated.

Callus Regeneration Stage

Through this stage, the effect of cytokinine kind and concentrations were conducted. Two kinds of cytokinin, Zeatin (Z) and 6-benzyl amino purine (BAP were

evaluated different concentrations (0-0.5-1.0-1.5) mg/L for callus regeneration (Table 2-D). Calluses were subcultured every four weeks, on the same fresh media, for three times. Cultures were incubated at light intensity of 30 μMm^{-2} S^{-1} for 16 h photoperiod at 24 ± 1.0 °C. Calluses were subcultured after four weeks to fresh solidified regeneration media. Percentage of callus regenerated and the percentage of shoots formed by callus were calculated. Regenerated adventitious shoots about 1–2 cm long were excised, transferred to MS medium (Table 2-A), and subcultured every four weeks. Rooted shoots were transferred to pots containing peat-moss, in green house for acclimatization. Survival of acclimatized were evaluated after 30 days.

Table 2. Composition of protoplast medium.

Media Composition	Initial and Multiplication mg/L) (Medium A	protoplast culture (mg/L) Medium B	callus induction (mg/L) Medium C	Callus Regeneration (mg/L) Medium D
Salt Minerals	MS	MS*	MS*	MS*
Thiamin	1.0	0.25	-	-
Pyridoxine	-	0.25	0.5	0.5
Nicotinic acid	-	2.5	0.5	0.5
Folic acid	-	0.25	0.5	0.5
Biotin	-	0.01	0.05	0.05
Glycine	-	1.0	2.0	2.0
Casein-Hydrolysate	-	250	400	1000
Myo-Inositol	100	50	100	100
Silver thiosulfate	50 µm	-	-	-
Glutamine	-	-	100	146
Sucrose	30 g/l	0.5 g/L	2.5 g/L	2.5 g/L
Mannitol	-	72800 -54600	40000	30000
Adenine sulfate	-		40	80
MES	-	500	976	976
NAA	-	0.5	0-0.1-0.25-0.5	-
IAA	-	-	-	0.1
BAP	-	-	0.5	0-0.5-1-1.5
Z	-	0-0.5-1-1.5	-	0-0.5-1-1.5-2
GA3	-	0.1	-	-
Agar	7.0 g/L	--	7.0 g/L	7.0 g/L
pH	5.8	5.8	5.6	5.6

MS* Mineral salts of MS (1962) modified

ISSR analysis

DNA was extracted from 5 potato *in vitro* regenerated plantlets and their parent plant using the CTAB procedure (Murray and Thompson, 1980). Eighteen primers obtained from VBC Company were used in this study (Table 3). Amplified of DNA was performed by standard PCR (APOLLO, USA).

Statistical analysis

Data were subjected to ANOVA analysis, and means were estimated of two experiments. Mean values were compared according to least significant differences test (LSD) at 1% probability. The obtained results were statistically analyzed by using SPSS analysis system (Statistical Package for the Social Sciences, ver. 17 for windows 2007).

RESULTS AND DISCUSSION

Initial Culture

After 30 days of culture, shoot tips produce microshoots (7-10 cm in length). Plant samples microcuttings (1-2 cm) and transferred to the same medium (Table 2-A), in order to increase the number of shoots which came from rose ends culture of tubers of two potatoes cultivars "Binella" and "Burren". Shoots were multiplied several times in order to obtain a sufficient material necessary for conducting of protoplast isolation and regeneration experiments.

Table 3. Primers sequence used in this study.

Primer	Sequence
ISSR1	(CT)8TG
ISSR2	(CT)8AC
ISSR3	(CT)8GC
ISSR4	(CA)6AC
ISSR5	(CA)6GT
ISSR6	(CA)6AG
ISSR7	(CA)6GG
ISSR8	(GA)6GG
ISSR9	(CAC)3GC
ISSR10	(GAG)3GC
ISSR11	(CTC)3GC
ISSR12	(GTG)3GC
ISSR13	GAG(CAA)5
ISSR14	CTG(AG)8
ISSR15	(AG)8TG
ISSR16	(ATG)5
ISSR17	(AG)8
ISSR18	(CCA)5

Protoplast Isolation and culture

Protoplast isolation from the leaves of potato cultivars "Binella" and "Burren", was earlier described by Al Qabbani *et al.* (2012). The supplement of 50 µm of silver

thiosulfate (STS), to the propagation medium (Table 2-A) increase leaf area, decrease the internodes length and increased the density and viability of isolated protoplasts, in both cultivars "Binella" and "Burren". The yield of protoplasts 1.93×10^6 protoplasts/g of fresh weight of leaves, with a viability of 84.9% for cv. Binella, and 1.44×10^6 protoplasts/g with a viability of 82.6% for cv. Burren. Protoplast divisions started 4 days for the cv. Binella, and 5 days for the cv. Burren, with the supplement of 1-1.5 mg/L of Zeatin. This dose not agree with previous finding by Thomas (1981), who stated that the start of cell division in potato cv. MarisBard and Mark Piper was after3-4 days when using 0.5 mg/L of Z. On the other hand, protoplast grwoo on hormone-free medium did not undergo any division and turned brown and died. Maximum plating efficiency obtained on medium supplemented with 1.0 mg/L Z in the cv. "Binella" and "Burren" (1.58 ± 0.1% and 1.27 ± 0.09 %) respectively, with significant differences between both cultivars. This similar to previuose finding by Kikuta *et al*. (1984), where the percentage cells forming colonies was 5% in cv. May Queen when using Z concentration of 1.0 mg/L. On the other hand Tican (1996), reoprted maximum percentage of cell colonies was 18.3% in cv. Desiree when using Z with 0.5 mg/L and the ratio was 0.48% at the concentrations of 1.0 mg/L. Higher protoplast plating efficiencies were also recorded in cv. Binella as comparedto cv. Burren because different genotypes most probably have specific nutritional or hormonal requirements to initiate, and/or sustain division of their protoplasts (Anjum, 1998).

Callus Intiation Stage

Table 4 shows that the highest percentage of callus initiation from the cell colonies, obtained when using 0.1 mg/L NAA growth regulator for cv."Binella" and "Burren" (51.12 ± 8.77% and 18.96 ± 2.81%) respectively, with significant differences between the two cultivars. No callus formation was observed when using hormone free Medium. This result agrees with Tican (1996) where the percentage of Callus formation in cv. Desiree was 20.8% with the supplement of 0.1 mg/L NAA. This is similarly to previous finding by Ehsanpour and Jones (2001).

Table 4. Effect of different naphthalene acetic acid (NAA) concentrations on callus initiation of potato protoplast culture

NAA concentration (mg/L)	Callus initiation (%)	
	Binella	**Burren**
0	0f	0f
0.1	51.12±8.77a	18.96±2.81d
0.25	41.31±3.99b	16.68±2.33d
0.5	31.22±2.11c	12.22±2.98e

Means with the same letter are not significantly different at P = 0.01.

Regeneration Stage

The initiated callus was transferred to the regeneration medium (Table 2-D). Results in table (5) indicated that Z was more suitable for plant regeneration than BAP for cv. Binella. Maximum callus formation was obtained on medium D with the supplement of 1.5 mg/L Z, which was 81.25 ± 6.25%, with significant difference in comparison to the same concentration of BAP, where the percentage of buds new formed was (43.75 ± 6.25%). Similar results were obtained on potato cv. Maris Bard that Z was more effective than BAP on plant regeneration from callus derived protoplasts where the percentage of neo formed buds constituted 50% on a medium containing 0.5 mg/L of Z (Thomas, 1981). Maximum shoot formation per callus in cv. Binella was obtained on medium D (1.5 mg/L Z). No shoots were formed on medium supplemented with BAP. Previous finding by Gary et al. (1985) showed that 71% shoot formation when using Z was used. While study by

Ehsanpour and Jones (2001) reported 30% shoot formation on medium supplemented with 1.0 mg/L Z. Moreover, previous study by Sadohara (1993), showed that Z is more effective than BAP in shoots regeneration, these probably due to the fact that hormone Z is a naturally cytokinin, which made its absorption by plant cells easier, while BAP is a synthetically growth regulators.

Table 5. Effect of different concentration of Zeatin and 6-benzyl amino purine (BAP) concentrations on protoplast regeneration from callus of potato cvs."Binella" and "Burren"

Growth regulators concentrations (mg/L)	Formation of new callus buds (%)		Mean number of new formed shoot per callus	
Zeatin				
	Binella	Burren	Binella	Burren
0	0^{d}	0	0^{c}	0
0.5	25 ± 0.00^{c}	0	0.13 ± 0.08^{b}	0
1	58.33 ± 8.33^{b}	0	0.25 ± 0.14^{b}	0
1.5	81.25 ± 6.25^{a}	0	1.06 ± 0.33^{a}	0
2	73.75 ± 6.25^{bc}	0	0.31 ± 0.15^{b}	0
BAP				
0.5	25 ± 0.000^{c}	0	0^{c}	0
1	33.33 ± 8.33^{c}	0	0^{c}	0
1.5	43.75 ± 6.25^{bc}	0	0^{c}	0

Means with the same letter are not significantly different at P = 0.01.

Table 6. Plant regeneration from potato protoplast cv."Binella".

Time per week	Media used	*In vitro* development states
4	Protoplast culture medium B + 1 mg/L Z +0.5 mg/L NAA+ 0.1 mg/L GA_3.	- cell wall regeneration - protoplast division -cells colonies formation
8	Callus initiation medium C + 0.1 mg/L NAA + 0.5 mg/L BAP.	-microcallus formation -callus in size 2-3 mm
12-16	Plant regeneration medium D +1.5 mg/L Z + 0.1 mg/L IAA.	-bud new formation
24-28	Plant multiplication medium A	-shoot elongation and rooting
28-36	Acclimatization in green house	-young plants in green house with mini-tubers productions

ISSR Analysis

Eighteen primers were tested to analyze 5 regenerated plantlets as well as the mother plant. Table 7 shows that, regenerated plants were 100% similar to mother plant (Figure 2). The results show plants that were not varying DNA level. Our results coincide with Ehsanpour and Jones (2001), where the plants regeneration from protoplast culture in the cultivar Delaware is primarily similar to mother plant. On the other hand, Gary *et al*. (1985) showed that 57% of regeneration plants from protoplast cv. Majestic got the same number of chromosome of the mother plant (n = 4x = 48). There are numerous reports in the literature about **c**hanges in both chromosome number and structure in plants regenerated from potato protoplasts. One of the common changes (aneuploidy) has been reported for many varieties for example, Russet Burbank and Fortyfold (Shepard, 1982)

Considering the small size of potato chromosomes it is likely that the ones described here represent a small fraction of those present in protoplast- derived plants, and that many more occur which cannot be easily identified (Lambert and Geelen, 2010). In the current study, simple conditions for isolation, culture and regeneration of plants from protoplasts of potato cvs. " Binella" and "Burren" were describe. The protocol described allows reproducible regeneration of plants from protoplasts of these cultivars and possibly other potato cultivars.

Table 7. Number of polymorphic bands and percentage of variation in 5 regenerated plantlets and their parent in cv. Binella.

Primers	Bands number	Variation%
ISSR1	5	**0**
ISSR2	7	**0**
ISSR3	3	**0**
ISSR4	8	**0**
ISSR5	5	**0**
ISSR6	4	**0**
ISSR7	7	**0**
ISSR8	4	**0**
ISSR9	7	**0**
ISSR10	4	**0**
ISSR11	6	**0**
ISSR12	7	**0**
ISSR13	7	**0**
ISSR14	7	**0**
ISSR15	7	**0**
ISSR16	3	**0**
ISSR17	5	**0**
ISSR18	8	**0**

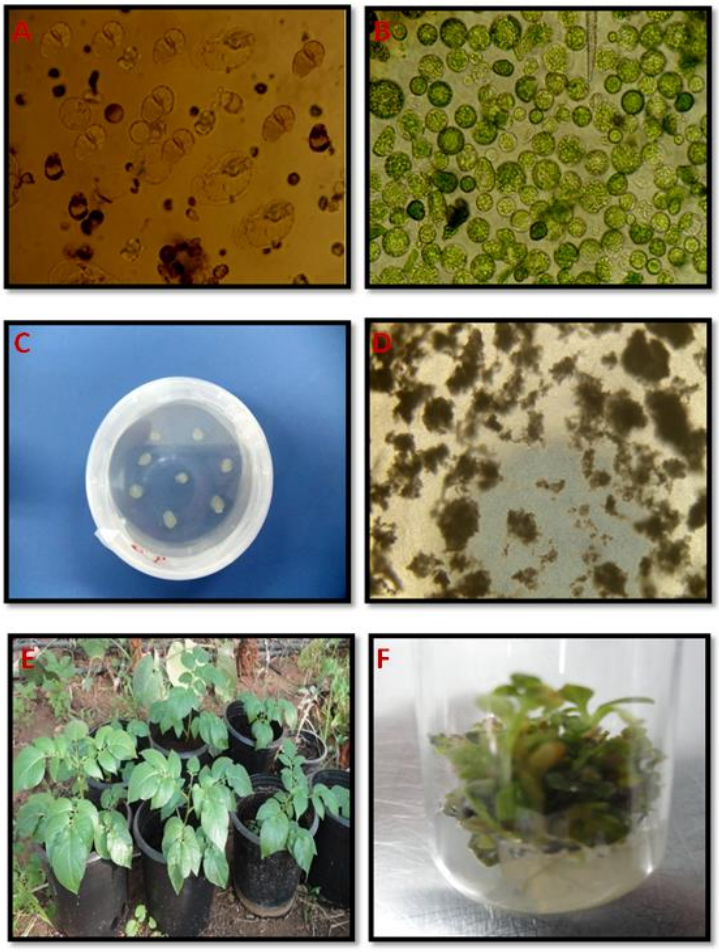

Figure 1. Potato protoplasts regeneratiom (cv. Binella).

A). Fresh isolated mesophyll protoplasts. B). Cells undergoing the first division in culture. C). Cell colonies after 30 days of culture. D). Callus formation after 8 weeks. E). Callus with plantlets. F). Regenerated plant grown in the glass house.

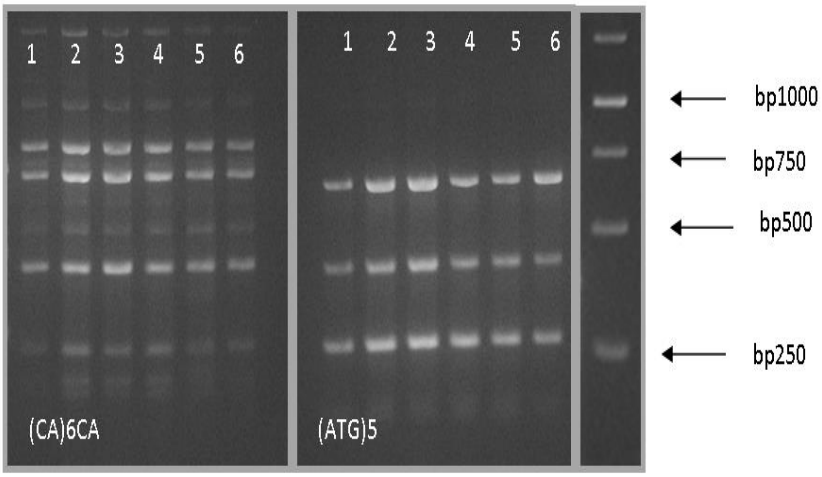

Figure 2. ISSR profile of potato regenerated plantlets and parental plant, DNA Molecular weight 1000bp, line 1: parental plant, lines 2 to 6: regenerated plants.

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عزل البروتوبلاست في البطاطا وتجديده بصنفين بينلا وبورين

المعري[1] والقباني[2] والبيسكي[2]

ملخص

هدف الدراسة الحالية الى تطوير طريقة سهلة وسريعة لعزل وزرع وتجديد البروتوبلاست من أوراق صنفي البطاطا بنيلا وبورين. حددت أفضل الشروط لزرع البروتوبلاست واستحداث الكالوس وتجديده، وكذلك تم تحديد مدى التشابه الوراثي بين النباتات المتجددة والنبات الأم باستخدام تقنية التكرارات التتابعية التسلسلية البسيطة (*ISSR*). عزلت البروتوبلاست من أوراق البطاطا المزروعة مخبرياً، التي تم الحصول عليها من نموات بعمر 4 أسابيع مزروعة في وسط الإكثار المزود بثيوسلفات الفضة بتركيز 50 ميكرومول. بدأت البروتوبلاست بالانقسام بعد *4* أيام في الصنف بنيلا، و بعد *5* أيام في الصنف بورين عند استخدام وسط الزرع *B*(*Murashig and Skoog, 1992*) والمزود بالزياتين (*Z*) بتركيز *1* و *1.5* مغ/ل، ولوحظ أعلى معدل لتشكل المستعمرات الخلوية عند استخدام *Z* بتركيز *1* مغ/ل وذلك بفروق معنوية بين الصنفين بنيلا وبورين (*0.1±1.58%* و *0.09±1.27%*) على التوالي. وتم الحصول على أعلى نسبة لتجدد النموات من الكالوس عند استخدام نفتالين أسيتك أسيد *(NAA)* بتركيز *0.1* مغ/ل وبفروق معنوية بين الصنفين بنيلا وبورين (*8.77±51.12%* و *2.81±18.96%*) على التوالي، و بلغت أعلى نسبة لتشكل النموات الخضرية من كالوس الصنف بنيلا *(17.68%±50)*، عند استخدام وسط تجديد يحتوي *1.5* مغ/ل من *Z*، في حين لم يتم الحصول على أي نمو من كالوس الصنف بورين. بلغت نسبة نجاح عملية التقسية *92*% بعد شهرين من نقل النباتات إلى البيت البلاستيكي، وبلغ متوسط طول النباتات *26.6* سم، وتم الحصول على درينات متوسط قطرها *13.17* مم ومتوسط وزنها *2.06* غ. بيّنت تقنية *ISSR* أن النباتات المتجددة من كالوس الصنف بنيلا كانت مماثلة تماماً للنبات الأم بنسبة *100*%.

الكلمات الدالة: بطاطا، مانيتول، مماثل للأصل، التكرارات التتابعية التسلسلية البسيطة، ثيوسلفات الفضة، زياتين..

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1) كلية الزراعة، جامعة دمشق، دمشق، سورية.

2) اللجنة الوطنية للتكنولوجيا الحيوية، دمشق، سورية.

kmaarri@yahoo.com

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