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Production of Virus Free Potato Plants Using Meristem Culture from Cultivars Grown under Jordanian Environment

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Abstract: Meristem culture has become a powerful and successful tool for virus elimination from infected plants and has been successfully applied in potato. A total of 70 potato (*Solanum tuberosum* L.) tubers for each of three cultivars that grown in Jordan were obtained from the Ministry of Agriculture and subjected to enzyme-linked immunosorbent assay (ELISA) test for detection of virus infection. The percentage of infection with *Potato virus Y* (PVY) was 21.4%, 15.7 %, 12.8% for Spunta, Alaska and Safrane cultivars, respectively. Meristem culture was applied on infected potato tubers for all the three cultivars. Results of shoots and roots development indicated that medium supplemented with 0.5 mg/l of Indole butyric acid (IBA) was the best for shoots length with mean (7.71 cm), roots length with mean (9.41 cm), number of shoots with mean (2.60) and number of leaves with mean (15.40). ELISA results for *in vitro* produced plantlets showed that all tested plantlets were PVY free. Reverse transcription polymerase chain reaction (RT-PCR) was successfully amplified the coat protein gene of PVY virus in the infected samples and confirm the ELISA result. The Acclimatization of the plantlets that generated from *in vitro* shoots and roots multiplication stage revealed 90% of successful with Spunta cultivar and 80% with both Alaska and Safrane cultivars.

Key words: *(Solanum tuberosum L.)* · Potato meristem culture · ELISA test · RT-PCR test

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the world's important food crops; it is grown in large areas around the world [1] and ranks the fourth importance after rice, wheat and maize [2]. Potato is an herbaceous dicotyledonous plant that propagated vegetatively through tubers; it is a native to South America. Potato is used for human consumption, animal feed and as source of starch and alcohol. Potato is considered among the economically important crops that grown in Jordan; statistics revealed that the total cultivated area was 17270 dunum and the annual production was 59230 tons during 2010 [3].

Virus and viroid diseases are among the major significant disease in potato seed production and certification. They include: *Potato leaf roll virus* (PLRV), *Potato virus A* (PVA), *Potato virus M* (PVM), *Potato virus S* (PVS), *Potato virus X* (PVX), *Potato virus Y* (PVY) and *Potato spindle tuber viroid* (PSTVd) [4]. The most common viruses affecting potato throughout the world are (PVY), (PVX) and (PLRV) [5, 6]. The presence of viral disease is an important reason attributed to low yield of potato varieties; the yield reduction may be up to 75% caused by the infection of some viruses. As such PVX alone may cause yield reduction of 15-30%; PLRV and some strains of PVY frequently reduce tuber yield by 50-80% [7].

Plant tissue culture in Jordan had witnessed a significant advance in the last decades; various research projects in plant biotechnological fields, preservation of plant genetic resources and the production of virus-free plants are standing examples [8]. Meristem tissues are virus-free because the phloem and xylem vessels are not present in meristem [9]. Meristem tissue culture has been successfully applied in potato for development of virus free plants and appeared a new venture in obliging virus free potato tuber seeds [10]; an increase of 40% in potato yield could be obtained after virus free tubers were used. Potato virus free clones with meristem culture methods were also practiced by [11, 12]. Meristem culture along with thermotherapy has become a powerful and successful tool for virus elimination such as PVX, PVY and PLRV from infected plants [13, 14].

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In plant diagnostic work, the use of enzyme-linked immunosorbent assay (ELISA) test and the use of molecular techniques have increased in recent years, enabling a sensitive detection of plant pathogens necessary for virus certification programs. The ELISA technique has become a standard method for the detection of plant viruses and has been applied with special success to the detection and identification of potato viruses [15]. The reverse transcriptase- polymerase chain reaction (RT-PCR), which is based on detection of one pathogen, is a simple method and widely used for detecting RNA viruses [16]. RT-PCR tests combining a cDNA synthesis and PCR amplification have been described for the detection of potato viruses like PVY, PLRV and PVA [17].

High cost of potato seed tubers, handling, storage and spread of virus diseases, in particular, are among the major problems of potato production in Jordan [18]. Therefore, the objectives of this study were to develop a reproducible meristem culture protocol for the production of virus free potato plants that grown under Jordanian environment and to use both ELISA and RT-PCR techniques for confirming the absence of viruses in the potato plantlets that developed by meristem culture.

MATERIALS AND METHODS

Plant Materials: Tubers of three commercially grown potato varieties (Alaska, Spunta and Safrane) were obtained from the Ministry of Agriculture in Jordan and used in this study. To test for virus infection, seventy tubers from each cultivar were subjected to double antibody sandwich DAS-ELISA. Virus infected tubers were chosen and used for sprouts production. All antisera were purchased from (ADGEN Co.). The standard direct DAS-ELISA was performed as described by [19] for detection of PVY.

Establishment of Meristem Culture: Thirty PVY infected sprouts from tubers of each variety were used as explants for meristem culture. The excised sprouts were sterilized in 0.1% hypochlorite and 3 drops of Tween-20 for 10 minutes, followed by 3 times washing with sterilized distil water. The tip and sub-tending leaf primordial were removed and the meristems were isolated under laminar air flow cabinet using dissecting microscope. Murashige and Skoog medium [20] free of hormone was used as basal culture medium. After adjusting the pH to 5.7, the medium was solidified with 8 g/l of Difco Bacto agar and autoclaved at 121°C for 30 min. The isolated meristems were quickly transferred to sterilized Petri dishes containing 10 ml MS medium. Cultures were kept at 25±2°C and 16/8 light/dark photoperiod in the culture room. The size of the meristem was about 0.3 mm at the time of culture.

Shoots and Roots Development: After 4 weeks of meristem culture, 10 of the developed meristems from each cultivar were sub cultured on MS hormone free medium and 10 on MS medium supplemented with (0.5 mg/l) of Naphthalene acetic acid (NAA) and 10 on MS medium supplemented with (0.5 mg/l) of Indole butyric acid (IBA). All the sub cultures were conducted on 15 ml of MS medium (pH 5.7) that solidified with 8 g/l of Difco Bacto agar, dispensed in Pyrex test tube (25x150 mm) and then autoclaved at 121°C for 30 min. Cultures were incubated in the culture room at 25±2°C and 16/8 light/dark photoperiod; one kilo lux light intensity was provided by using cool-white fluorescent tubes. After 5 weeks, developed plantlets were examined for various parameters: shoot length, root length, number of leaves, number of roots and number of shoots.

Acclimatization: Ten *In vitro* rooted plantlets from each cultivar were removed from the test tubes and thoroughly cleaned from agar with sterile distilled water. The plantlets were transferred to small pots (7cm diameter) containing 1:1 peat moss: perlite mixture and covered with plastic bag. The pots were kept at 25 °C, (16/8) light/dark photoperiod for 4 weeks in the culture room. After that, the plantlets were transferred to the green house and continue growing at 26±2°C.

Reverse Transcriptase-Polymerase Chain Reaction (RT- PCR): The total RNA was extracted from leaves, taken from potato plantlets grown inside the green house, using the EZ-10 Spin Column Total RNA Minipreps Super Kit (Bio Basic, Canada) according to manufacturer' instructions. The extracted RNA was used as template for cDNA synthesis. A fragment of PVY coat protein with expected size of 801 bp was amplified with a pair of specific primers:

- PVYCPv *BamH1* (5'TCAAGGATCCGCAAATGACA CAATTGATGCAGAGG-3') and PVYCPc
- *EcoR1* (5'-AGAGAGAATTCATCACATGTTCTTGA CTCC-3'). The coat protein (CP) of PVY was amplified using the access RT-PCR system (Promega, USA) according to the manufacturer's instructions.

Detection of Amplified PCR Products: Agarose gel electrophoresis was performed as described by [21]. Aliquots 10 µl of each PCR products were resolved electrophoretically on a 1% agarose gel using 0.5X TBE buffer. The PCR products were visualized under UV transilluminator and photographed with gel documentation system (Gel Doc 200, BIO-RAD, USA) after staining the gel with ethidium bromide (0.5µg/ml). 1 kb DNA ladder (Promega, USA) was used as a marker to determine the size of the amplified fragments.

Statistical Analysis: The recorded data were subjected to the analysis of variance (ANOVA). Least Significant Differences (LSD) at probability <0.05 were used to asses the differences among means.

RESULTS

Results of DAS-ELISA test showed that PVY was detected in 15 potato tubers out of 70 (21.4%) that examined from Spunta cultivar, 11 potato tubers out of 70 (15.7%) from Alaska cultivar and 9 potato tubers out of 70 (12.8%) from Safrane cultivar. The infected tubers from each cultivar were used as a source for meristems culture.

Thirty meristems out of 90 were developed from the three cultivars that cultured on hormone free medium. The meristems commenced their initial growth by increasing in size and gradually changed to light green color; within 2-3 weeks small leaves appeared. After four weeks of incubation, the 30 meristems that divided equally to three media (10 for each) were developed to plantlets that represent the three cultivars. All the *In vitro* developed potato plantlets were subjected to DAS-ELISA test and were free of virus contaminations. The developed plantlets were studied for five characters: shoots length, roots length, number of shoots, number of roots and number of leaves. The cultivars, media and the interactions between cultivars and media showed statistically significant differences for all the five studied characters (Table 1).

Shoots Length: Spunta cultivar showed the highest mean (6.88 cm) followed by Safrane (4.89 cm) and Alaska (4.52 cm). Shoots length was the highest on medium containing 0.5 mg/l IBA with mean 7.71 cm followed by medium containing 0.5 mg/l NAA with mean 6.04 cm. The shortest shoots length was observed on hormone free medium (control) with mean equals to 2.54 cm as shown in Table (2). For interaction between cultivars and media, the highest shoots length was 9.30 cm for Spunta cultivar on medium with 0.5 mg/l IBA, while the shortest shoots length was obtained at hormone free medium with mean equals 2.11 cm for Alaska cultivar.

Roots Length: As shown in Table (2), the highest response of roots length was in Spunta cultivar with mean equal to 7.88 cm followed by 5.53 cm and 4.83 cm for Alaska and Safrane cultivars respectively. Medium containing IBA was found to be the most effective and produced the longest roots length with Spunta cultivar (9.41 cm) followed by medium containing NAA with mean equals to 7.31 cm. For interaction, the roots length ranged from 1.22 cm at hormone free medium with Alaska cultivar to 12.87 cm at medium containing IBA with Spunta cultivar.

Number of Shoots: The means of shoots number for cultivars Spunta, Alaska and Safrane were 2.66, 1.93 and 1.53 respectively. Medium supplemented with IBA was found to be the most effective one with mean 2.60, while hormone free media was the least effect and produced 1.36 shoots. For interactions, highest number of shoots (3.60) was observed at the 0.5 mg/l IBA followed by 0.5 mg/l NAA with mean 2.80 with Spunta cultivar. The lowest mean number of shoots (1.10) was observed in Alaska cultivar at hormone free medium (Table 2).

Number of Roots: The mean values presented in Table (2) indicated that Spunta cultivar produced the highest number of roots (5.40) followed by Safrane and Alaska with means equal to 4.07, 3.87 respectively. No significant differences were observed between media containing

Table 1: Mean squares and degree of freedom (d. f.) from the analysis of variance for the effects of three media on five characters of the plantlets developed from meristems of three potato cultivars

Source of variations	d.f.	shoot length	root length	number of shoots	number of roots	number of leaves
Cultivar (C)	2	48.37*	76.85*	9.91*	20.84*	151.60*
Media (M)	2	208.3*	501.78*	11.74*	394.67*	1428.70*
C X M	4	13.28*	27.67*	1.87*	10.69*	42.95*
Error	81	0.835	6.34	0.58	4.37	7.04

* Significant at $\alpha = 0.05$

Table 2: Means of five characters for three cultivars and three media

	Shoots length				Roots length				Number of shoots				Number of roots				Number of leaves			
Cultivars	Control	IBA	NAA	Means	Control	IBA	NAA	Means	Control	IBA	NAA	Means	Control	IBA	NAA	Means	Control	IBA	NAA	Means
Spunta	3.20	9.30	8.15	6.88a	1.42	12.87	9.36	7.88a	1.60	3.60	2.80	2.66a	1.00	10.40	4.80	5.40a	2.70	19.30	14.90	12.30a
Alaska	2.11	5.73	5.72	4.52b	1.22	8.42	6.96	5.53b	1.10	1.60	1.90	1.53b	0.90	7.60	3.10	3.87b	2.40	11.30	10.00	7.90b
Safrane	2.33	8.10	4.26	4.89b	1.91	6.95	5.62	4.83bc	1.40	2.60	1.80	1.93c	1.40	6.90	3.90	4.07b	2.40	15.60	14.70	10.90c
Means	2.54a	7.71b	6.04c	-	1.52a	9.41b	7.31c	-	1.36a	2.60b	2.16c	-	1.10a	3.87b	4.07b	-	2.50a	15.40b	13.20c	-

IBA and NAA but they differed significantly with hormone free medium. For interaction between cultivar and media, the highest means (10.40) was observed on IBA medium with Spunta cultivar followed by Alaska with mean 7.60, while Alaska gave the lowest mean value (0.90) for number of roots with the control treatment.

Number of Leaves: For cultivars, Alaska produced the lowest mean (7.90) for number of leaves, while Spunta produced the highest (12.30) followed by Safrane with mean equal to 10.90 (Table 2). The results also indicated that medium containing IBA was the most effective with mean 15.40 comparing to medium containing NAA with mean 13.20 and hormone free medium with mean 2.50. For interaction, the highest number of leaves was observed with Spunta cultivar (19.30) followed by Safrane (15.60) at IBA medium, while Alaska and Safrane at hormone free medium gave the lowest mean for number of leaves (2.40).

Acclimatization: Plantlets from the three cultivars were gradually acclimatized. Potato plantlets pots were covered with plastic bags, the bags were gradually removed within two weeks. After four weeks, plantlets developed and their leaves increased in size. 90% survival plantlets were produced from Spunta cultivar and 80% from Alaska and Safrane cultivars. The plantlets were transferred to the green house in which they continue growing.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR): Ten plantlets from each cultivar were subjected to RT-PCR. The RT-PCR products were visualized under UV transilluminator following electrophoresis through a 1% agarose gel in 0.5X TBE buffer. RT-PCR for plasmid (positive control) and PVY infected potato plants leaves produced bands of the expected size (801bp). The results of RT-PCR produced from *in vitro* plantlets for the three cultivars revealed the absence of PVY virus in the plantlets that developed by meristem culture (Figure 1).

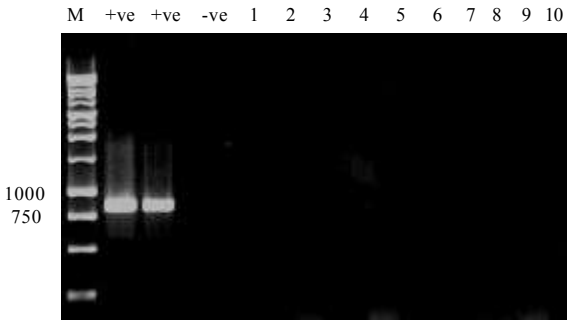

Fig. 1: Detection of PVY in Spunta cultivar by RT-PCR. Lane M 1Kb DNA Marker, lane 2 positive control, lane 3 PVY infected sample, lane 4 negative control, lanes 5-14 Spunta potato plantlets developed from the meristem culture.

DISCUSSION

Potatoes seem to harbor many viruses in Jordan very often in mixed infection. For the purpose of this study, tubers infected only with PVY as demonstrate by serology were used as a source of meristem culture. The correct diagnosis of any viral disease is a prerequisite of control; the more rapidly and accurately the causal organism is identified, the sooner the proper controls can be instituted. Apathy toward research on control of viral disease is due to large of difficulties in identifying the viruses. The use of symtomology and other biological testes to detect and identify PVY with large number of samples was not entirely practical, in term of numbers of plants and space needed lead to confusion particularly in mixed infections. Also, the tests were often lacking of accuracy, difficult to interpret and required weeks or months to complete. Therefore, the use of other methods of virus detection including serology and molecular was essentially needed.

Meristem of three widely cultivated varieties in Jordan namely Alaska, Spunta and Safrane were established on hormone free MS medium in order to produce potato plant PVY free. The developed meristems were sub cultured on MS medium supplemented with 0.5

mg/l NAA and 0.5 mg/l IBA and on hormone free MS medium. The results of this study showed that the medium supplemented with 0.5 mg/l IBA was the best medium for shoots length with mean (7.71), roots length with mean (9.41), number of shoots with mean (2.60) and also the best for the number of leaves with mean (15.40). Using of 0.5 mg/l IBA was most effective for proper shoot and root development from primary developed meristem [22].

Spunta and Safrane cultivars were more responsive for micropropagation of plantlets than Alaska. Different response of different potato varieties due to genetic make up towards *in vitro* shoot multiplication and their development were reported by [23, 22].

The results of this research showed that all potato plantlets samples from the three cultivars derived from meristem culture that subjected to ELISA and RT-PCR were free of PVY virus; the results of this study are in agreement with the findings of [24, 13, 22]. The results of RT-PCR confirmed the absence of PVY virus in the potato plantlets developed by meristem culture, primers; PVYCPv *BamH1*, PVYCPc *EcoR1* were be efficient to detection of PVY in the infected sample by producing band with size 801 bp and this results was in conformity with [5].

This study also demonstrates the value of meristem culture to free potato from an important virus, PVY. Previous investigations have suggested that the elimination of virus by tip culture could be due to the failure of virus to enter the meristem [25-27].

Potato is generally propagated and distributed as vegetative material. Several important vascular pathogens, including viruses and virus like disease, are systemic in diseased plants and are difficult to detect unless special indexing tests are used. Because of the high risk of introducing new pathogens to Jordan in the transfer of planting material, it may be possible, however, to promote the international exchange of potato by shipping pathogen-free plants in the form of meristem tip cultures.

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