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Meristems culture for virus irradiation in potato (*Solanum tuberosum*) cultivars in Palestine

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ABSTRACT

Potato production is highly affected by viral diseases such as potato virus X (PVX) and potato virus Y (PVY) which can be overcome by establishing a reproducible meristem culture protocol for the production of virus-free potato plants. This study was conducted during 2017 and 2018 on two potato cultivars "Spunta" and "Alaska Red" in Palestine. The tubers were subjected to reverse transcriptase-polymerase chain reaction (RT- PCR) for detection of virus infection. The infected tubers for both cultivars were incubated, and after sprouting, meristems-tips were isolated and cultured on Murashige and Skoog (MS) medium. After four weeks, developed shoots were cultured on MS media supplemented with different rates of three hormonal combinations (0.54 µM NAA; 0.54 µM NAA + 1.0 µM TDZ; 0.54 µM NAA + 2.2 µM BA; 0.54 µM NAA + 2.3 µM Kinetin; 0.54 µM NAA + 1.0 µM TDZ+ 2.2 µM BA + 2.3 µM Kinetin). The results showed that maximum shoot multiplication was observed when NAA at 0.54 µM was combined with 1 µM TDZ (2.85 and 2.55 shoots) in both "Spunta" and "Alaska Red" cultivars, respectively. Higher shoot length and leaf number were obtained when NAA was combined with either BA (2.2 µM) or Kinetin (2.3 µM) in both cultivars. *In vitro* multiplied shoots were rooted and acclimatized successfully. Neither t PVY nor PVX viruses were detected by RT-PCR on the RNA extracts from the tested *in vitro* grown samples.

Key words : Meristem culture, potato, potato virus X, potato virus Y, RT-PCR

INTRODUCTION

Potato (*Solanum tuberosum* L.) ranks among the topmost important food commodities in the world (FAO, 2014). Because of its high nutritional value, productivity, and ability to adapt to various climate conditions, potato is grown in many countries (Zheng *et al.*, 2020; Bakunov *et al.*, 2022). According to the cultivated area, potatoes stand the fourth in the world (Al-Taleb *et al.,* 2011). In Palestine, according to 2014 statistical data, the total potato cultivated area was 1650 ha with an annual production of about 53000 tons (FAO, 2014), Many Mediterranean countries including Palestine import large amounts of certified potato tubers. However, locally produced tubers are mainly used for planting in autumn and winter, while the imported tubers are used for early and late spring plantings.

Tubers' quality is considered an important character for potato yield. Viruses and other diseases infect potato plants during cultivation, causing plant degradation and a decrease in crop yield (Grishchenko *et al.*, 2021). Potato leaf roll virus (PLRV) and Potato virus Y (PVY) are considered two of the most damaging viruses of potatoes and are epidemic in most potato cultivation areas Potato virus Y is reported to be the most important virus that affected potato production, mostly in the autumn plantings, within the Mediterranean countries. Potato leafroll virus is coming after PVY, damages up to 90%. Of the crop (Loebenstein and Gaba, 2012). Different attempts were reported to eliminate viruses, among these attempts were in vitro meristem culture, that results of viral-free potato plantlets yield high-quality sanitized seed material (Grishchenko *et al.*, 2021).

In vitro propagation methods using

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meristem tips and micro tubers are considered more reliable for maintaining the genetic integrity of the multiplied clones (Badoni and Chauhan, 2009; Dawinder et al., 2020; Dmitrieva et al., 2021). In vitro chemotherapy in combination with electrotherapy (20 mA for 20 min) produced 50.0 % elimination of - Cyrtanthus elatus virus-A from Narcissus plants. Thermotherapy followed by meristem culture resulted in 90% elimination of PLRV and 93% of PVY viruses, while meristem culture alone resulted in only 56 and 62% elimination of PLRV and PVY, respectively (Wang et al., 2006). In vitro thermotherapy at approximately 37oC, for 40 days resulted in 37.5% PVY elimination. However, chemotherapy resulted in 55.5% elimination (Nascimento et al., 2003). Similar results were also reported by Dmitrieva et al. (2021). However, it was found that in vitro electrotherapy resulted in 100 % BBTV-free valuable clones from the infected banana material (Singh et al., 2022). The enzyme-linked immunoassay (ELISA) technique is considered the standard method for the detection of plant viruses including potato viruses (Shojaei et al., 2009). The reverse transcriptase-polymerase chain reaction (RT-PCR) is a simple method and widely used for detecting viruses. RT-PCR tests have been described for the detection of potato viruses such as PVY (Klerks et al., 2001), expression stability of candidate reference genes (Chandna et al., 2012) and recently for molecular diagnosis of COVID-19 (Feng et al., 2020).

Al-Taleb *et al.* (2011) and Renev *et al.* (2021) reported that meristem culture can be used as a useful method to produce PVY free plants from infected tubers. Although, there have been different attempts for *in vitro* production of virus-free potato plantlets until now no significant results were obtained. Therefore, the objective of this study was to establish a reproducible meristem culture protocol for the production of virus-free potato plants in Palestine.

MATERIALS AND METHODS

Field Surveys and Plant Materials Collection

During the winter/spring growing season of 2017 and 2018, field surveys were carried out in areas where potatoes are usually planted in Nablus (Latitude: 32°13′15″ N, Longitude: 35°15′15″ E, Elevation above sea level: 569 m) and Tubas (Latitude: 32°19′15″ N, Longitude: 35°22′11″ E, Elevation above sea level: 375 m) districts. Virus and virus-like diseases were inspected. Tubers from two commercially grown potato cultivars “Spunta” and “Alaska Red” were then obtained from the field to be used in this study. Fifty tubers from each cultivar were subjected to viral tests using the RT-PCR detection technique for putatively infected viruses. Then, tubers infected with viruses were chosen and used for sprouts production.

Sprouting and Meristem Culture Establishment

Twenty infected tubers of each cultivar were cleaned with running tap water, then dried and wrapped with 70% alcohol before being incubated in a growth chamber at 23°C and 16 h/8 h (light/dark) at an irradiance of 40 µmol/m^2/s. After 3 weeks, sprouted buds were separated from the tubers and treated with sodium hypochlorite (0.2% (V/V)) solution for 15 minutes, followed by 30-second washing with 70% alcohol, followed by three times washing (5 minutes/time) with sterile distilled water. The tips and sub-tending leaf primordials were removed and the meristems were isolated under a laminar airflow cabinet using a dissecting microscope. Next, the isolated meristems (0.2-0.3 mm) were cultured on a solid hormone-free MS media (Murashige and Skoog, 1962), and kept in a growth chamber at 22 ± 1°C and 16 h/8 h (light/dark) at an irradiance of 40 µmol/m^2/s (Fig. 1).

Shoot Multiplication Experiment

After 4 weeks of meristem culture, 50 *in vitro* meristems of both cultivars were transferred to the cultivation tubes containing the multiplication media Murashige and Skoog (MS) prepared according to (Abu Qaoud, 2013). The MS media was supplemented with different rates of three hormonal combinations consisted of: (1). 0.54 µM NAA, (2). 0.54 µM NAA + 1.0 µM TDZ, (3). 0.54 µM NAA + 2.2 µM BA, (4). 0.54 µM NAA + 2.3 µM Kinetin and (5). 0.54 µM NAA + 1.0 µM TDZ+ 2.2 µM BA + 2.3 µM Kinetin.

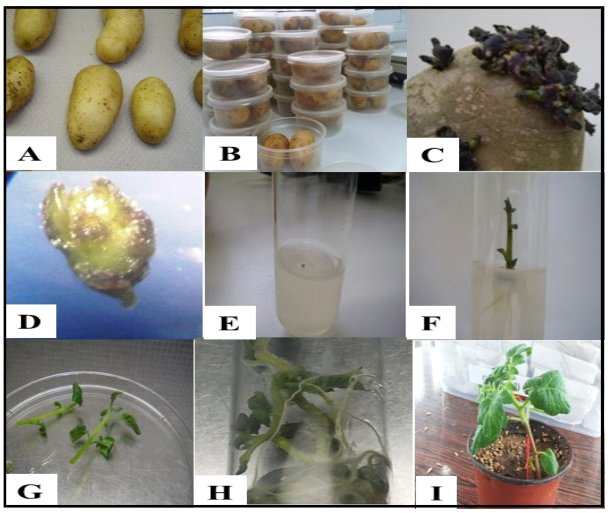

Fig. 1. A: Potato tubers of "Spunta" cultivar after cleaning. B: Incubated tubers. C: Sprouting tuber. D: Isolated meristem of "Spunta". E: Cultured meristem. F: Developing meristem. G & H: Multiplied shoots of "Spunta" cultivar. I: Acclimatized growing shoot of "Spunta".

The tubes were incubated at 22 ± 1°C and 16 h/8 h (light/dark) at an irradiance of 40 µmol/m^2/s. After four weeks, the number of shoots, shoot length and number of leaves per replicate were recorded. The experiment was repeated twice, and the average of both experiments was used for statistical analysis.

Rooting and Acclimatization

Appeared shoots (approximately 3-4 cm long) were cut out and their bases were dipped with T8 (IBA rooting powder) and then were transplanted to 10 cm in diameter pots filled with sterile vermiculite and peat moss mixture (1:2; v/v). The pots were sealed using thin plastic sheets and irrigated with sterile water; they were then kept under the previously mentioned growing conditions. One week later, the sheets were removed gradually. Shoots with well-formed roots were transplanted into 30 cm in diameter pots and kept in a greenhouse under partial shading (30-40% of shading).

Viral Testing

Collected potato samples were tested for viral infections using virus-specific primers for PVX and PVY. Sets of primers used to detect PVX designed as per standard method, meanwhile primer sets for detection of PVY were designed to be able to detect the PVY coat protein with the amplicon size 801 bp (Table 1) as mentioned by Al-Taleb *et al.* (2011).

Extraction of Total Nucleic Acids and cDNA Synthesis

RNA was extracted from freshly collected samples using the RNA Easy Extraction Kit (QIAGEN). First-strand cDNAs were synthesized using Moloney Murine Leukemia Virus reverse transcriptase (Life Technologies Ltd) (Alkowni and Abu-Qaoud, 2016).

Polymerase Chain Reaction (PCR) and Gel Electrophoresis Analysis

PCR mix was prepared by adding 2 µL cDNA to 2 µL sense Primer (2.5 µM); 2 µL antisense Primer2 (2.5 µM); 5 µL 10x Thermophilic DNA buffer; 4 µL $MgCl_2$ (25 mM); 1 µL dNTPs (10mM); 2.5 µL Taq DNA; polymerase (2u\µL) and the total volumes were adjusted to 50 µl by adding dH_2O. Then the mixture was put in PCR machine for 35 cycles at the following parameters: (94°C / 30 sec; 55°C / 30 sec; 72°C / 1 min) with final extension at 72°C / 10 min. PCR products were analyzed according to Alkowni and Abu-Qaoud (2016).

Data Analysis

Data were analyzed using SAS software (SAS Institute, 1990). ANOVA was conducted followed by mean separation using Duncan's Multiple Range test (DMR) at 0.05 probability level and numbers were presented as averages.

Table 1. RT-PCR primers designed to amplify PVY and PVX.

Virus	Primer name	Sequence (5'-3')	Amplicon size
PVY	CPv BamH1	TCAAGGATCCGCAAATGACACAATTGATGCAGAGG	801 bp
	CPc EcoR1	AGAGAGAATTCATCACATGTTCTTGACTCC	
PVX	Sense	TAGCACAACACAGGCCACAG	562 bp
	Antisense	GGCAGCATTCATTTCAGCTTC	

RESULTS AND DISCUSSION

Shoot Multiplication Experiment

Molecular data obtained from RT-PCR using the specific primers showed that "Spunta" and "Alaska Red" tested tubers were infected with PVY. Infection was 26% and 20% in "Spunta" and "Alaska Red" respectively. The meristems cultures were prepared from the infected tubers from both cultivars. After four weeks of incubation on hormone-free media, the growing meristems were developed into shoots that represent the two cultivars. All the *in vitro* developed shoots were subjected to RT-PCR test and were free of virus contaminations.

The results obtained for eliminating two of the most devastating potato viruses (PVX and PVY from infected tubers by meristems tip culture) agreed with previous trials carried on different potato cultivars (Truskinov and Rogozina, 1997; Mahmoud *et al.*, 2009). Besides that, this research study reported for the first time the spread of two viruses (PVX and PVY) in Palestinian fields planted with imported cultivars (Spunta and Alaska Red). Using the molecular tools (RT-PCR) which are considered more sensitive than ELISA in detecting the virus was also reported.

The effect of different hormones on shoot multiplication of "Spunta" and "Alaska Red" potato cultivars are presented in Table 2. Significant differences were observed in the average number of shoots multiplied per explants in response to the different hormone combinations and concentrations. Maximum shoot number (2.85 and 2.55) was observed when NAA was combined with 1 µM TDZ in both "Spunta" and "Alsaka Red" cultivars, respectively (Table 2).

Whereas shoot number was decreased significantly when other combinations were added. No multiplication was observed without hormone (control) and with NAA alone as well as when the three cytokinins were combined with NAA. All growth regulator combinations except (control, NAA alone, and all cytokinins) gave significantly higher shoot length that ranges between 7.0 to 7.48 cm in "Spunta", however, in "Alaska Red" both BA and Kinetin gave the highest shoot length (Table 2).

Lower shoot length with NAA treatment in "Spunta" and NAA and the control in "Alaska Red" were observed. A similar result was obtained when the three cytokinins were combined with NAA in both cultivars. A similar trend was observed with the average leaf number per shoot. A higher significant leaf number was obtained in both cultivars when each cytokinin was combined with NAA separately. The maximum number of leaves was observed with Kinetin (10.27 and 12.55) in both "Spunta" and "Alaska Red", respectively (Table 2).

Rooting, Acclimatization and Virus Tested Propagating Potatoes

A high percentage of survival plants (90%) were produced from both cultivars (data not shown). Samples produced through meristem cultures were subjected to RT-PCR tests for the PVX and PVY. Using the two steps RT-PCR, the potato virus Y was detected in many potatoes collected from the fields. The same result was obtained for PVX. All the *in vitro* developed shoots from the multiplication experiment after they were subjected to *in vitro* sanitation by meristems culturing were tested

Table 2. Effect of different hormones on shoot multiplication of "Spunta" and "Alaska Red" potato cultivars.

Treatments (µM)	Average shoot number		Average shoot length (cm)		The average number of leaves/shoot	
	"Spunta"	"Alaska Red"	"Spunta"	"Alaska Red"	"Spunta"	"Alaska Red"
Control	1.00 c	1.00 d	4.28 b	3.58 bc	6.57 bc	7.50 b
0.54 NAA	1.00 c	1.12 cd	3.42 bc	3.00 c	4.71 c	5.50 b
0.54 NAA + 1.0 TDZ	2.85 a	2.55 a	7.00 a	5.50 b	9.00 ab	6.25b
0.54 NAA + 2.2 BA	1.85 b	2.00 b	7.42 a	8.25 a	10.57 a	11.52 a
0.54 NAA + 2.3 Kinetin	1.28 b	1.50 cd	7.86 a	7.50 a	10.27 a	12.55 a
0.54 NAA + 1.0 TDZ + 2.2 BA + 2.3 Kinetin	1.00 c	1.00 d	2.71 c	3.50 c	4.86 c	6.50 b
Sig. level (P value)	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

Means in the same column followed by the same letters are not significantly different ($P < 0.05$).

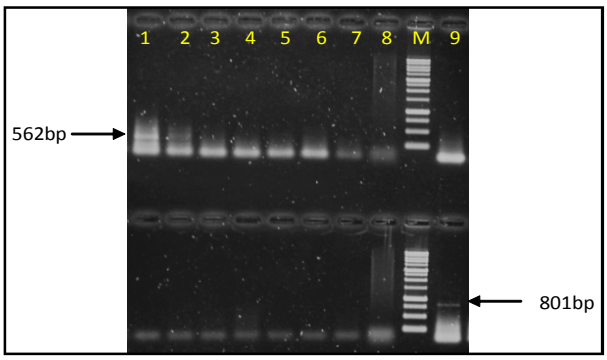

Fig. 2. The 1% gel electrophoresis for *Potato virus* X (PVX) shown up and *Potato virus* Y (PVY) were only detected with virus-infected potato tubers (1,2 & 9) from fields; while none of these viruses were found in meristems-tip cultured ones (3-8). 1kb markers were used.

for both viruses separately. All the tested samples were found free from both viruses (Fig. 2).

Field surveys and RT-PCR tests showed that planted potatoes are hosting viruses in the fields of Nablus and Tubas. It was noticed that potatoes exhibit viral symptoms in the fields while many of them were found symptomless (unpublished data). Field surveys covered the main areas of Nablus and Tubas districts revealed low exhibitions of Potato varieties to viral infections. Even though, some spots were noted to manifest leaf yellowing and stunting. No doubts that any virus infection foci will be the source to spread the virus through insect vectors (Davis *et al.,* 2005). In addition, the spread of the virus could be through propagated materials, thus disseminating healthy potato tuber is one of the top priorities of any certification program. In such a tale, sanitation methods besides propagating resistant varieties are inevitable. Compared to other protocols (Wang *et al.,* 2006), this experimental study, successfully eliminated (100%) of the PVY and PVX using meristem culture, therefore, meristem cultures of the two widely cultivated potato varieties in Palestine was established, the low meristem development in this study was in agreement with the results reported by Al-Taleb *et al.* (2011).

In this study, excised meristems were grown into multiplication media, maximum shoot multiplication was observed when NAA at 0.54 µM was combined with 1 µM TDZ in both cultivars. Higher shoot length and leaf number were obtained when NAA was combined with either BA (2.2 µM) or Kinetin (2.3 µM), this result was in agreement with Badoni and Chauhan, (2009) who found that potato shoot height was improved when Kinetin and NAA were used at 1 mg/L Kinetin and 0.1 mg/L NAA combination, as well as with the results reported by Ghaffoor *et al.* (2003) and Abd Elaleem *et al.* (2009). Variation between cultivars was also observed by (Hajare *et al.*, 2021) who reported that culture initiation was obtained on MS medium supplemented with 1.5 mg/l BAP + 3.0 mg/l NAA for Gudiene variety, whereas 1.0 mg/l BAP and 2.0 mg/l NAA produced more shoots in Belete potato variety, other researchers reported the good shoot regeneration of potato plant from callus and meristem by using the, BAP and NAA at the rate of 3.0 mg/L and 2.0 mg/L, respectively (Pawar *et al.*, 2019), while other reported the inclusion of Jasmine acid among both BAP and NAA highly improve potato shoot regeneration (Kumlay *et al.*, 2021).

In this study, using TDZ combined with NAA gave the best multiplication result compared to other cytokinins for both cultivars. This result was also in agreement with Dokhaniyeh *et al.* (2011) who found a superior rate of callus induction and plant regeneration from internode and petiole of potato explants when thidiazuron was used compared to zeatin, therefore, our result confirms the strong TDZ activity. TDZ has been demonstrated to have a high cytokinin metabolism on shoot regeneration of different plant species (Abu-Qaoud, 2012). TDZ is considered to be more potent than most of the commonly used cytokinins (Huetteman and Preece, 1993). In this study, different cytokinins were tried to induce shoot multiplication. It has been reported that exogenous application of different cytokinins (BA, KIN, TDZ, ZEA, etc) is an obligation for the induction of multiple shoots in many plants (Kishor and Devi, 2009).

CONCLUSION

In the present study, TDZ and NAA combination was found to be the best for shoot multiplication of Spunta and Alaska Red potato varieties with MS medium. Both cultivars exhibited a similar response to cultural conditions. This study also demonstrates the

value of meristem culture to free potatoes from an important virus (PVY).

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