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Obtaining PVX, PVY and PLRV-Free Micro Tuber from Granola, Pasinler 92 and Caspar Potato (*Solanum tuberosum* L.) Cultivars

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Abstract: This study was conducted to obtain virus-free propagation materials from Granola, Pasinler 92 and Caspar potato (*Solanum tuberosum* L.) cultivars infected with potato virus X (PVX), potato virus Y (PVY) and potato leaf roll virus (PLRV) by using meristem-tip culture. For *in vitro* propagation, it was tested the effect of different combinations and concentrations of benzylamino purine (BA) (0.0, 0.25, 0.50 mg L^{-1}) and gibberellic acid (GA_3) (0.0, 0.25, 0.50 mg L^{-1}) on the number of shoot and node. On the other hand, it was evaluated the effect of BA (0.00, 5.00, 10.0 mg L^{-1}) and CCC (chlorocholine chloride) (0.00, 500 mg L^{-1}) on the tuberization under two photoperiodic regimes (light and dark). The MS salts and vitamins supplemented with 30 g L^{-1} sucrose was used as a medium and the media was solidified with 7.0 g L^{-1} agar and the ratio of sucrose added into media for micro tuber production had been increased from 3-8%. The highest number of shoots was obtained from 0.00/0.25, 0.25/0.50 and 0.00/0.00 mg L^{-1} BA/GA_3 treatments for Granola, Pasinler 92 and Caspar cultivars as 1.52, 1.24 and 1.44, respectively. However, the highest number of node were determined on 0.00/0.50 for Granola (9.12), Pasinler 92 (8.76) and on 0.00/0.25 mg L^{-1} BA/GA_3 treatments for Caspar (8.24). When the results were assayed according to total tuber number, the most micro-tubers for Granola, Pasinler 92 and Caspar cultivars were obtained from 5.00/5000 mg L^{-1} BA/CCC treatment as 5.6, 4.0, 4.8 per/bottle under dark treatments. All *in vitro* regenerated plant materials were tested by DAS-ELISA (double antibody sandwich enzyme-linked immunosorbent assay) to determine the presence and absence of viruses and PVX, PVY and PLRV viruses were eliminated from Granola (25, 40 and 60%), Pasinler 92 (16, 41.6 and 46.1%) and Caspar cultivars (28.5, 33.3 and 50%), respectively.

Key words: Potato, virus elimination, *in vitro* propagation, micro-tuber production

INTRODUCTION

The potato (*Solanum tuberosum* L.) as a vegetatively propagated crop is prone to accumulative infection by bacteria, fungi, viruses and viroids a process commonly referred to as degeneration[1]. Virus diseases have been recognized as a limiting factor in potato production worldwide. More than 25 different viruses are known to naturally infect the potato. The most important viruses causing severe diseases in potato are potato leaf roll virus (PLRV), potato virus Y (PVY), potato virus S (PVS) and potato virus X (PVX). Other viruses may cause significant economic losses, but these have a restricted distribution[2]. In efforts to reduce extensive yield losses due to viral diseases in subsequent potato crops is the use of certified virus-free tuber as "seed" for planting[3].

Obtaining quantities of clean planting material has been a major barrier to increased potato production in many developing countries. Seed tuber multiplication is slow and diseases tend to increase with each multiplication. Therefore, developing countries frequently depend on new seed stock from developed countries each year[4,5]. For many years, tissue culture has been applied to improve potato production by means of micro propagation, pathogen elimination and germplasm conservation[6]. Micro propagation allows rapid multiplication of potato clones in a short duration under disease-free, controlled environment and on a year round basis[7]. On the other hand, micro propagated plants, when cultured under suitable conditions, produce *in vitro* micro-tubers[8-11]. When micro-tubers and micro propagated plants are planted in soil, they produce mini tubers. *In vitro* production of micro tubers has been used for mass multiplication of nuclear seed stock, storage and transport of germplasm[7,12]. However, large-scale commercial multiplication facilities required predictable multiplication rates if labor and resources are to be optimized.

Potato is one of the most important crops in Turkey. Total potato cultivated area of Turkey is 200 thousand ha

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and total potato tuber production is approximately 4-5 millions tons. Turkey needs approximately 125-150 tons potato seed per year and there is no state company that produce diseased-free seed potato in Turkey. Therefore, to meet the demand of farmers, seed potatoes have been imported, multiplied and then have been distributed to the producers by some private companies. Thus, it is required the use of tissue culture methods both in production of certified seeds being virus-free and the other pathogens and to conserve cultivars adapted environmental condition of Turkey.

This study was conducted to 1) determine the effect of different combination and concentration of BA and GA_3 on *in vitro* clonal propagation, 2) to evaluate the effect of BA and CCC for micro tuber production under two photoperiod (light and dark), 3) to obtain viruses-free (PVX, PVY and PLRV) starting material and to determine the ratio of elimination percentage of these viruses from Granola, Pasinler 92 and Caspar cultivars infected with PVX, PVY and PLRV.

MATERIALS AND METHODS

Granola, Pasinler 92 and Caspar cultivars used as plant materials in experiments were obtained from Agricultural Research Institute of East Anatolia, Erzurum, Turkey.

Dormancy of tubers were broken by incubating at 4°C for 120 days and surface sterilized by immersing in a 5% sodium hypochlorite solution containing 0.1% wetting agent (Tween-20) for 10 min. They were then washed twice with sterile distilled water and transferred into sterile petri dishes containing sterile filter paper for a short time to dry under the laminar air flow cabinet. Meristem-tips excised from potato sprouts, with one or two primordial, maximum 0.3 mm length were excised with sterile needle and razor blades under the binocular dissecting microscope (Wild M, Magnification 20-40) and then placed in test tubes (150x25 mm) containing 10 ml of the liquid Murashige and Skoog[13] salt solution, vitamins and 30 g L^{-1} sucrose for shoot initiation. For *in vitro* clonal propagation and micro tuber production, the media was solidified with 7.0 g L^{-1} agar and the ratio of sucrose added into media for micro tuber producing had been increased from 3 to 8%. In all media used in this study, pH was adjusted 5.7 and then was autoclaved at 121°C for 15 min.

After filtered, different combination and concentration of BA (0.0, 0.25, 0.50 mg L^{-1}) and GA_3 (0.0, 0.25, 0.50 mg L^{-1}) were tested to determine the best suitable treatment for *in vitro* propagation (individual four nodes with one leaf excised from virus-free plantlets placed on 200 ml bottles containing 10 ml medium and assayed each vessel as one replication) and the cultures were maintained at 24°C under 16/8 (light/dark) of fluorescent lights, at 2000 lux intensity. In order to determine the best treatment for the micro-tubers production, the effect of the combination and concentration of BA (0.00, 5.00, 10.0 mg L^{-1}) and CCC (0.00, 500 mg L^{-1}) under light and dark were tested. For this purpose, virus-free plantlets derived from meristem-tips (each with 10 nodes) were placed on horizontally on 400 ml bottles containing 20 ml culture medium.

Ten bottles were used for each treatments and half of these were exposed to under light (8/16 light/dark, at 1000 lux intensity, at 18°C) and the other five bottles were wrapped in aluminum foil to exclude light and kept under the same temperature conditions. The results were evaluated according to the number of shoot and node for *in vitro* clonal propagation, the total tuber number for micro-tuber production after 4 and 12 weeks, respectively. In all these experiments, the hormone-free MS medium was assigned as a control and the experimental design was a completely randomized. All treatments were performed with five replications and data were subjected to analysis of variance (ANOVA) and were separated Duncan's Multiple Range Test[14] in the SPSS program[15].

DAS-ELISA was used to determine the absence or presence of PVX, PVY and PLRV at each step. As a criteria, in order to determine the viruses elimination percentage from cultivars, it was only used plantlets developed from meristem-tips not die. ELISA assay kits was brought from Boehinger Mannheim Company (Germany) and was performed as described previously[16].

RESULTS AND DISCUSSION

The effect of BA and GA_3 on the number of shoot and node: It was shown that there were significant differences among treatments, cultivars and treatments x cultivars interactions concerning the average number of shoots and nodes ($p<0.01$).

The highest number of shoots was obtained from 0.00/0.25, 0.25/0.50 and 0.00/0.00 mg L^{-1} BA/GA_3 treatments for Granola, Pasinler 92 and Caspar cultivars as 1.52, 1.24 and 1.44, respectively (Table 1). However, the highest number of node was obtained from 0.00/0.50 for Granola, Pasinler 92 and 0.00/0.25 mg L^{-1} BA/GA_3 treatments for Caspar as 9.12, 8.84 and 8.24, respectively. It was observed that the application of BA with GA_3 or without prevented rotting, increased calli formation, decreased the number and size of leaves. Moreover, the

Table 1: The effect of BA and GA_3 on the number of shoot and node under for Granola, Pasinler 92 and Caspar cultivars

Cultivars	Granola		Pasinler 92		Caspar	
BA/GA_3 (mg L^{-1})	Number of shoot	Number of node	Number of shoot	Number of node	Number of shoot	Number of node
0.00/0.00	1.32±0.18b	8.24±0.33b	1.00±0.00d	6.60±0.20c	1.44±0.17a	7.60±0.70a
0.25/0.00	1.08±0.11c	4.60±0.24f	1.00±0.00d	3.92±0.27g	1.12±0.11cd	4.20±0.32d
0.50/0.00	1.04±0.94c	4.32±0.23f	1.00±0.00d	3.44±0.33h	1.08±0.11cd	4.28±0.23d
0.00/0.25	1.52±0.10a	8.84±0.39a	1.04±0.94cd	8.08±0.30b	1.32±0.18ab	8.20±0.20a
0.00/0.50	1.44±0.94ab	9.12±0.36a	1.12±0.10a-d	8.76±0.26a	1.20±0.14bc	7.96±0.26a
0.25/0.25	1.04±0.10c	5.20±0.32de	1.16±0.94a-c	5.32±0.36e	1.08±0.11cd	5.96±0.26c
0.50/0.50	1.00±0.10c	5.28±0.48d	1.08±0.11b-d	4.48±0.30f	1.00±0.00d	6.12±0.33c
0.50/0.25	1.00±0.10c	4.76±0.38ed	1.20±0.14ab	4.12±0.24g	1.04±0.90cd	5.88±0.30c
0.25/0.50	1.12±0.10c	6.64±0.52c	1.24±0.17a	5.88±0.23d	1.16±1.94b-d	6.76±0.26b

a, b, c, d: Means for each column with the same letter(s) are not significantly different ($p<0.01$)

Table 2: The effect of BA and CCC on the micro tuber formation under light and dark photoperiod for Granola, Pasinler 92 and Caspar cultivars

Hormon treatments						
Under Light (16/8 light/dark)						
Cultivars	0.0/0.00	0.0/500	5.0.00	5.0/500	10.0/0.00	10.0/500
Granula	3.8±0.84a	3.0±1.00ab	2.4±0.55	2.6±0.55b	02.2±0.84b	03.2±0.84b
Pasinler 92	2.2±0.84bc	3.2±0.84a	1.6±0.55c	2.0±0.70bc	01.4±0.55c	02.8±0.45ab
Caspar	3.6±0.55a	2.8±0.84a-b	1.8±0.45d	2.4±0.55b-d	02.0±0.70cd	03.2±0.84ab
Under Dark						
Cultivars	0.0/0.00	0.0/500	5./0.00	5.0/500	10.0/0.00	10.0/500
Granula	4.8±1.30ab	3.2±0.84cd	4.2±0.84a-c	5.6±1.14a	3.8±1.09bc	2.2±0.84d
Pasinler 92	3.6±1.14ab	2.8±0.45b	3.0±0.70ab	4.0±0.70a	3.2±0.84ab	1.6±0.90c
Caspar	4.0±1.00ab	3.4±1.14bc	3.2±0.84bc	4.8±0.84a	3.8±0.84ab	2.4±0.90c

a, b, c, d: Means for each rows with the same letter(s) are non significant

Table 3: The elimination percentage of PVX, PVY and PLRV viruses from Granola, Pasinler 92 and Caspar cultivars

Cultivars	Viruses	No. of excised meristem-tips	Developed plantlets	Virus-free plantlets	The Elimination % age
Granola	PVX	20	4	1	25.0
	PVY	48	15	6	40.0
	PLRV	29	10	6	60.0
Pasinler 92	PVX	20	6	1	16.0
	PVY	36	12	5	41.6
	PLRV	32	13	6	46.1
Caspar	PVX	20	5	2	28.5
	PVY	31	9	3	33.3
	PLRV	33	10	5	50.0

increasing of BA concentration in the medium increased callus size and caused growing tiny and weak of shoots. In the hormone-free and medium including only GA_3, all of the plantlets rotted, better developed and no calli formation. On the other hand, plantlets treated with only GA_3 internodes were too long, thin and fragile.

Similar results were reported previous studies[17-20]. This results suggesting that BA and GA_3 could not added propagation medium unless required. Because, micro propagated plants can be direct transfer into soil to produce mini-tuber. It is required that plantlets must be healthy and rooted in that case[7]. Therefore, cytokinin and oxin hormones were not added either or added in fewer concentrations to *in vitro* propagation media. On the other hand, similar case is valid for GA_3 as well[21-23].

The effect of BA and CCC on the formation of micro tuber under light and dark: It was found that there were significant differences among treatments, light, cultivar and treatments x light interactions at significant level on the number of micro tuberization. However, treatment x cultivar, light x cultivar, treatment x light x cultivar interactions had no significant effect on tuberization. On the other hand, light, treatment, cultivar, treatment x cultivar, light x cultivar and treatment x light x cultivar interactions were found to have significant effect on the weight of micro tuber formation ($p<0.01$).

The number of micro tubers varied with the cultivars, BA/CCC and photoperiodic treatments. When the results were assayed according to total tuber number, the most micro-tubers for Granola, Pasinler 92 and Caspar cultivars were obtained from 5.00/5000 mg L^{-1} BA/CCC treatment as 5.6, 4.0, 4.8 per/bottle under dark treatments (Table 2).

It was observed that tuberization began earlier in the darkness than in the light and light treatment stimulated shoot growth, branching and root growth regardless of cultivar or photoperiodic conditions on the hormones-free medium. Similar results were reported by Estrada *et al.*[10], Novak and Asiedu[24]. All micro tubers were cream colored independent from all treatments in the darkness. However,

the color of tubers was green and browns all treatments in the light. It was appeared that BA caused calli formation, strongly inhibited root formation in all treatments and decreased the number of tuber in the light but vice versa in the darkness.

Up to 10.00/500 mg L^{-1} BA/CCC concentrations in both dark and light conditions, the number of tuber decreased. Only CCC (500 mg L^{-1}) application in both light and dark period decreased the number of tuber and inhibited stem elongation. On the other hand, application of BA and CCC hormones (5.00/500 mg L^{-1}) increased number of tubers in the darkness compared to light period. For mini tuber production, the number of micro-tuber is more important than micro tuber weight. Because, mini tubers were produced from either micro tubers or micro propagated plants *in vitro*[7]. Therefore, micro-tuber numbers play favorable role in choosing of media type. Nevertheless, micro tuber production affected by particularly, cultivars[25], photoperiod[10,24,26-28], explants number, duration of *in vitro* culture, temperature, sucrose ingredient, amount of hormones and balance in media[7,8,11,24,25,29,30].

The percentage of viruses elimination rate from cultivars: Fifty tubers from each cultivar were tested with DAS-ELISA (from tuber sprout) to find out virus-free propagation materials and results were recorded. As a result of this study, it was found that tubers were determined to be infected with at least one of PVX, PVY and PLRV viruses and no virus-free material was found. The virus elimination percentage from cultivars (Granola, Pasinler 92 and Caspar) using the only meristem-tip culture was given (Table 3). It was appeared that the elimination rate of viruses varied among cultivars and viruses.

In conclusion, PVX, PVY and PLRV viruses were eliminated from Granola (25, 40 and 60%), Pasinler 92 (16, 41.6 and 46.1%) and Caspar (28.5, 33.3 and 50%), respectively.

The use of thermotherapy, chemotherapy and electrotherapy and meristem-tip culture has been used as a means of eradicating viruses. On the other hand, it was stated that the rate of virus eradication and survival of meristem-tips depend on virus, cultivar, the size of excised meristem-tips. When the size of meristem-tip increased, virus elimination percentage decreased or vice versa[31-34]. Therefore, when only meristem-tip culture was used for virus elimination, meristem-tips had been excised smaller than 0.3 mm[20]. On the other hand, when it was used together with thermotherapy, chemotherapy and electrotherapy, the meristem-tips had been cut higher than 0.5 mm[23,35-39].

The low virus elimination can be explained by attributed to the use of only meristem-tip culture. As meristem-tip length increased, meristem-tip development increased, whereas virus elimination ratio decreased or vice versa. Application of meristem-tip culture together with thermotherapy, chemotherapy and electrotherapy, requires more labor intensive work. However, it is difficult and requires special skills to cut meristem-tips less than 0.3 mm length. Therefore, researchers have to decide the type of methods needs to be used in their studies.

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