Scientific Papers. Series B, Horticulture. Vol. LXII, 2018
Print ISSN 2285-5653, CD-ROM ISSN 2285-5661, Online ISSN 2286-1580, ISSN-L 2285-5653

IN VITRO EFFECT OF GENOTYPE, GROWTH SEASON AND CYTOKININES ON PEACH VARIETIES (*Prunus persica* (L.) Batsch) PROPAGATION

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Abstract

In vitro propagation is one of the most convenient methods for plant material multiplication in order to obtain virus free planting material in high quantity and short time. The paper presents the influences of genotype, growth season and hormones on in vitro propagation of some new Romanian peach varieties with very good prospects on the market. Three peach genotypes: 'Florin', 'Filip' and 'Mimi' from the Didactic Field of Faculty of Horticulture, University of Agronomic Sciences and Veterinary Medicine of Bucharest were tested. In the protocol for the in vitro establishment, winter and spring growth season were chosen. Initial shoot explants were obtained in winter by forcing the dormant shoots in the growing chamber and in spring (April-May), directly from the field. The explants were cultured on MS (Murashige and Skoog, 1962) basal medium supplemented with 30 g sucrose and 7 g agar, as carbon source. Benzyl aminopurine (BAP) on four variants with 0, 1, 5 and, respectively, 10 mg/l was used as cytokinine. The results show that Florin genotype was superior to the rest of the varieties used in the experiment in terms of the number of formed shoots and the strength of their growth. For the in vitro culture initiation, the shoots taken in spring, during the growth season, gave the best results compared to the winter period. The results showed a significant correlation between the concentration of BAP and the shoots number (multiplication rate) and height. The concentration of 5 mg/l BAP (V3), gave the best rate of shoot formation and the highest elongation rate.

Keywords: *benzyl aminopurine, culture media, shoots, multiplication rate, elongation rate*

INTRODUCTION

Peach (*Prunus persica* (L.) Batsch) is one of the most popular stone fruits. Peaches belong to *Prunoideae*, *Prunus* genus a subfamily of *Rosaceae*. It is one of the most popular deciduous fruits and ranks second to apple among temperate zone deciduous fruit trees from the standpoint of production and value (Childers, 1978; USDA, 2017).

China is the centre of origin for peaches and nectarines and was domesticated there 4000 years ago (Wang and Zhuang, 2001). Peach fruits are of high nutritional value because they contain high levels of carbohydrates, fats, salts and vitamins and are used in the treatment of anaemia, poor digestion and nourishment of the nervous systems (Al-Sheikh, 2003). Micropropagation is one form of tissue culture which allows the production of large number of plants from small pieces of the mother plant in relatively short period of time and limited space. It is an aseptic process which requires sophisticated laboratory procedure with unique facilities and special skills (Hartmann et al., 2004; Sathyanarayana, 2007). Micropropagation is affected by many factors such as genotype, plant growth regulators (PGRs), agar, type of explants, culture medium and light conditions etc. For example, cytokinins are major factors in the induction of somatic organs (George, 1993; Feyissa et al., 2005).

Several experiments were carried out for the multiplication of wood plants by tissue culture such as Ferradini N. et al. (1996) on apple rootstock and Peticila A.G. (2012) on kiwi. In addition, the difficulty of regenerating plants from mature tissues of woody plants is well established (Smigocki et al., 1991).

Peach is one of the most recalcitrant species with regard to micropropagation (Bhansali et al., 1990; Padilla et al., 2006). Successful regeneration of peach plants were from immature seeds (Meng and Zhou., 1981; Hammerschlag et al., 1985; Scorza et al., 1990;

Bhansali et al., 1991; Smigocki et al., 1991; Svircev et al., 1993; Pérez- Clemente et al., 2004). Also regenerated from leaves explants excised from shoots apexs culture (Gentile et al., 2002).

Therefore, the main goal of this study was to establish a micropropagation protocol for 'Florin', 'Filip' and 'Mimi' peach varieties in order to produce a large scale of plants in a short period. Plant hormones are the most important effect factors in shoot regeneration (Bhojwani and Razdan, 1996). Cytokinins are a type of plant growth regulators (PGRs). Contributed to in many processes of plants growth, like cells division, Shoot and root morphogenesis. PGRs are regulating axillary bud growth. Considering the importance of these PGRs, among growth regulators used in peach tissue culture media are the cytokinins: TDZ, kinetin and BAP, for auxins: IBA, IAA and NAA (Hammerschlag, 1985; Mante et al., 1989). This study aimed to evaluate different concentrations of BAP cytokinins for *in vitro* shoot development in peach**.**

MATERIALS AND METHODS

The study was conducted at the tissue culture laboratory of the Faculty of Horticulture, University of Agronomic Sciences and Veterinary Medicine of Bucharest during the period October 2016 - June 2017 on Peach (*Prunus persica* L.). Three peach varieties ('Florin', 'Filip' and 'Mimi') were included in the experiment. Explants were taken from trees planted in the field of experiments to the Faculty of Horticulture, University of Agricultural Sciences and Veterinary Medicine in Bucharest. Two explants types namely shoot-tips and nodes (one node with a single axillary buds) were taken at 0.5-1cm length, were tested on their ability to maintain and initiate shoots on MS medium without any hormone supplements during initiation stage and added 0, 1, 5, respectively, 10 mg/l Benzyl aminopurine (BAP) during multiplication stage. V1=0 mg/l (control); V2=1 mg/l; V3=5 mg/l; V4=10 mg/l.

Shoot-tips and nodes were obtained from two sources (last year's growths, modern growths). The explants were taken in the winter season (dormancy buds) 15-20 cm long and placed in jars containing water to stimulate the sprouts to grow and break the dormancy (Figure 1). After two-three weeks were taken buds formed and used in the experiment, while the nodes were used directly in the experiment. In the growing season (spring: April-May) the explants were taken and used directly in the experiment.

Media and culture conditions

All explants were rinsed with ethanol 70% for 2-3 min then were washed with distilled water three times for 2-3 min, Explants were surface sterilized with NaOCl (10% v/v) for 10-15 min. Then explants were washed with sterile distilled water at least three times for 5 min. MS (Moorashige and Skoog, 1962) consisting of 30 g/l sucrose and 7 g/l agar without any hormone supplements during initiation stage and added 0, 1, 5, respectively, 10 mg/l Benzyl aminopurine (BAP) during multiplication stage. The pH of medium was adjusted to 5.6 with HCl 0.1 N or NaOH 0.1 N before sterilization by autoclaving at 121°C for 15 minutes. Sterilized explants were inoculated on culture media and then placed in an incubation room at 22±2°C, and 16 hours daily (Stănică et al., 2002).

Statistical analysis

The experiment was repeated two times, each treatment contained 10 replicates initiation stage and 5 replicates tested on multiplication stage. All experiments were arranged in a completely randomized design (CRD). Culture period ranged between four to eight weeks depending on individual experiment. Data were recorded on shoots number formed, shoots length and leaves number were analyzed. Significance of differences between the results was estimated by Analysis of Variance (ANOVA) on SPSS version 14 (SPSS 2005) program with the means compared with LSD test at < 0.05.

Figure 1. Peach branches placed in water for break dormancy

RESULTS AND DISCUSSIONS

Analysis of variance (Tables 1 and 2) revealed that the treatment had highly significant effect on mean leaves number and shoots length. The results of means mean leaves number and shoots length formed developing in response to all the varieties of the experiment for the tissue culture and there were significant differences between the varieties, where Florin variety had the largest number of leaves formed 5.00 leaf per explants in winter and 5.80 leaf per explants in spring while the 'Fillip' and 'Mimi' varieties gave the least number of leaves. Also the 'Florin' variety gave the longest shoots formed (2.37 cm in winter and 4.77 cm in spring) while the 'Filip' variety gave the shortest shoots formed (1.69 cm in winter and 2.77 cm in spring).

Figure 2. Hoot-tips of peach on MS after 2 week from culture

There were no significant differences in shoot number per explants in initiation stage. There was a single shoot in all explants (Figure 2). Growth of roots in the hormone-free medium was not observed. In multiplication stage also there were significant differences between the varieties. 'Florin' variety superior on 'Filip' and 'Mimi' varieties in the number and length of shoots formed (Figure 3). The effect of genotype on successful tissue culture has been previously reported (Gubis et al., 2003; Blinstrubiene et al., 2004). Cotton callus initiation (Zouzou et al., 1997; Zouzou et al., 2000) at all the genotypes were cultured onto hormone-free medium. It can be assumed that the differences in their response in tissue culture were determined by the balance of their endogenous hormones (Razdan, 2003). The difference might be due to intra-metabolism of plant which affected cell division and differentiation (Techato et al., 2002).

The results showed that there were statistical differences in the shoots length of and leaves number formed by shoot-tips and nodes. Results showed superiority shoot-tips on nodes (Tables 1 and 2). Results showed that each type of explants are characterized by a certain regeneration potential, depending on the species of plant and its degree of maturity, which is of physiological state of explants. Several types of explants have been widely used for *in vitro* such as Citrus lemon (Rathore et al., 2004); young leaves on French bean (Kamal and Praven, 1991); terminal buds of renewal on gladioli (Rumynin et al., 1990); apical buds on hybrid of mountain ash (Suvorova et al., 1990) and Rough lemon (Ali and Mizra, 2006).

Analysis of the results showed that there were significant differences between leaves number formed and shoots length from explants which were taken from different seasons (Table. 2). Explants taken in the growth season (spring) gave the best results, as opposed to explants which were taken in the winter for all varieties (Table.1).

Previous studies have confirmed that there is a relationship between phenolic compounds and the age of the plant used, a common problem reported in tissue culture of woody species (Mc Cown, 2000; Mathur et al., 1999). Ozyigit (2008) indicated a positive direct relationship between age of explants and phenolic exudation in tissue culture of cotton.

Different combinations of cytokinins (BAP) interacted significantly in terms of the shoots number (Table 3). Variant V3 (BAP 5 mg/l) gave the maximum shoots number ('Florin' 8.00, 'Filip' 7.40 and 'Mimi' 5.40). Lowest number of shoot (1.00) were obtained in control medium (without any plant growth regulators). Increasing BAP doses in combination had an increasing effect up to a certain level V4 (BAP10 mg/l). Data presented in higher shoots length were obtained in control medium (without any plant growth regulators).

This data shows that the shoot's lengths were markedly affected by various combinations of cytokinins. Statistically, after treatment V4 (BAP 10 mg/l) with elevated levels of cytokinins, shoot length decreased ('Florin' 2.59 cm, 'Filip' 1.77 cm and 'Mimi' 1.94 cm). Analysis of LSD values between varieties and variants showed that the effect of BAP on the shoots number and shoots lengths were significant at $p \leq 0.05$ (Tables 4 and 5).

Similar regeneration behaviours of BAP in pear (Kadata and Numi, 2003); in peach rootstock GF 677 (Ahmad et al., 2003); in bananas by (Vuylsteke, 1989; Arinaitwe et al., 2000). Previous researchers Vuylsteke and De Langhe (1985); Bairu et al. (2008) indicated that 5 mg/l BAP was the best concentration for banana varieties.

In vitro multiplication rate was largely controlled by interaction the varieties and cytokinins concentration and BAP is the most economical cytokinins (Gaspar et al., 1996; Augusto,. 2001; Silveira et al., 2009). Rapid growth and multiplication of shoots are based on the quantity and quality of cytokinins and auxins in media as well as on their endogenous levels in plants. Histological studies showed that the inclusion of BAP in shoot proliferation media enhanced the growth of axial shoots and promoted the multiplication of shoots from the basal tissues of explants (Ohki and Sawaki, 1999). A decline in the number of shoots with higher BAP levels has also been reported. Waseem et al. (2009) showed that the use of higher concentrations of PGRs may result in plant weakness and decreased growth (Panjaitan et al., 2007).

Al-Sulaiman and Barakat (2010) cytokinins had a positive effect on the production of lateral shoots of *Ziziphus spinachristi*. The appropriate addition of cytokinin promotes the growth of shoots and reduces the dominance of the apical bud (Asaad et al., 2009). In the *Prunus* species the type of citokinine and its concentration are important factors for multiplication and elongation rate (Leontiev-Orlov et al., 2000a). Increasing (BAP) causing a rise in the numbers of buds primordia in chrysanthemum (Karim et al., 2002, 2003). PGRs for bud break and shoot differentiation due to their role in cell multiplication and the breakdown of apical dominance (Casimiro et al., 2001). In woody plants, BAP is paramount for growth compared to kinetin (Fráguas et al., 2004).

A B C

Figure 3. Peach genotypes after 3 weeks on V3 (MS+ BAP 5 mg/l): 'Florin' (A); 'Fillip' (B); 'Mimi' (C)

Table 1. Analysis of Means and Std. Deviation for the effect of genotype, growth season and explants on leaves number and shoots length after 4 weeks cultivation on three peach varieties ('Florin', 'Filip' and 'Mimi')

Genotype	Season	Explants	Mean leaves number	Std. deviation	Mean shoots length	Std. deviation
Florin	Winter	Shoot-tips	5.0000	1.00000	2.3740	.80640
		Nodes	3.0000	1.22474	2.0180	.90502
		Total	4.0000	1.49071	2.1960	.82961
	Spring	Shoot-tips	5.8000	1.09545	4.7760	.92802
		Nodes	8.0000	2.34521	3.0220	.31260
		Total	6.9000	2.07900	3.8990	1.13172
	Total	Shoot-tips	5.4000	1.07497	3.5750	1.50813
		Nodes	5.5000	3.17105	2.5200	.82914
		Total	5.4500	2.30503	3.0475	1.30227
Filip	Winter	Shoot-tips	3.8000	.83666	1.6900	.48974
		Nodes	3.2000	1.30384	1.3960	.20671
		Total	3.5000	1.08012	1.5430	.38678
	Spring	Shoot-tips	4.4000	.54772	2.7700	.53810
		Nodes	4.6000	.54772	2.2960	.54344
		Total	4.5000	.52705	2.5330	.56776
	Total	Shoot-tips	4.1000	.73786	2.2300	.74786
		Nodes	3.9000	1.19722	1.8460	.61258
		Total	4.0000	.97333	2.0380	.69389
Mimi	Winter	Shoot-tips	2.6000	.54772	1.6140	.68090
		Nodes	2.4000	1.14018	1.2900	.29589
		Total	2.5000	.84984	1.4520	.52357
	Spring	Shoot-tips	3.8000	1.30384	2.9420	.40752
		Nodes	2.6000	.54772	1.9780	.13554
		Total	3.2000	1.13529	2.4600	.58319
	Total	Shoot-tips	3.2000	1.13529	2.2780	.87735
		Nodes	2.5000	.84984	1.6340	.42256
		Total	2.8500	1.03999	1.9560	.74722
Total	Winter	Shoot-tips	3.8000	1.26491	1.8927	.71550
		Nodes	2.8667	1.18723	1.5680	.61784
		Total	3.3333	1.29544	1.7303	.67726
	Spring	Shoot-tips	4.6667	1.29099	3.4960	1.12216
		Nodes	5.0667	2.65832	2.4320	.56753
		Total	4.8667	2.06336	2.9640	1.02771
	Total	Shoot-tips	4.2333	1.33089	2.6943	1.23283
		Nodes	3.9667	2.31164	2.0000	.72996
		Total	4.1000	1.87490	2.3472	1.06373

Table.2. Analysis of variance (ANOVA) for the effect of genotype, growth season and explants on leaves number and shoots length after 4 weeks cultivation on three peach varieties ('Florin', 'Filip' and 'Mimi')

Source	Dependent Variable	Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Leaves number	144.200[a]	11	13.109	9.956	.000
	Shoots length cm	50.635[b]	11	4.603	13.702	.000
Intercept	Leaves number	1008.600	1	1008.600	766.025	.000
	Shoots length cm	330.551	1	330.551	983.935	.000
Varieties	Leaves number	67.900	2	33.950	25.785	.000
	Shoots length cm	14.781	2	7.391	21.999	.000
Season	Leaves number	35.267	1	35.267	26.785	.000
	Shoots length cm	22.829	1	22.829	67.954	.000
Explants	Leaves number	1.067	1	1.067	.810	.373
	Shoots length cm	7.231	1	7.231	21.526	.000
Genotype *Season *Explants	Leaves number	17.433	2	8.717	6.620	.003
	Shoots length cm	.946	2	.473	1.408	.255
Genotype *Season	Leaves number	14.233	2	7.117	5.405	.008
	Shoots length cm	1.653	2	.826	2.460	.096
Genotype *Explants	Leaves number	1.633	2	.817	.620	.542
	Shoots length cm	1.145	2	.572	1.704	.193
Season *Explants	Leaves number	6.667	1	6.667	5.063	.029
	Shoots length cm	2.050	1	2.050	6.102	.017
Error	Leaves number	63.200	48	1.317		
	Shoots length cm	16.126	48	.336		
Total	Leaves number	1216.000	60			
	Shoots length cm	397.312	60			
Corrected Total	Leaves number	207.400	59			
	Shoots length cm	66.760	59			

Table. 3. Combined effect of genotype and concentrations of BAP on shoots number and shoots length per explants

Genotypes	Variants	Mean shoots number	Std. deviation	Mean shoots length (cm)	Std. deviation
Florin	V1	1.0000	.00000	5.7380	.67887
	V2	4.0000	.70711	3.8440	.69540
	V3	8.0000	1.87083	4.7760	.92802
	V4	3.8000	1.30384	2.5920	.91319
	Total	4.2000	2.78341	4.2375	1.40635
Filip	V1	1.0000	.00000	3.3340	.69346
	V2	2.4000	.54772	2.7700	.53810
	V3	7.4000	1.51658	3.1440	.62616
	V4	1.6000	.89443	1.7680	.43275
	Total	3.1000	2.73188	2.7540	.81770
Mimi	V1	1.0000	.00000	4.0980	.43390
	V2	2.2000	.83666	2.9420	.40752
	V3	5.4000	1.14018	3.8880	.44263
	V4	1.2000	.44721	1.9400	.69199
	Total	2.4500	1.93241	3.2170	.99420
Total	V1	1.0000	.00000	4.3900	1.18345
	V2	2.8667	1.06010	3.1853	.71137
	V3	6.9333	1.83095	3.9360	.94388
	V4	2.2000	1.47358	2.1000	.75069
	Total	3.2500	2.57514	3.4028	1.24945

Table 4. LSD values between genotype on shoots number and shoots length per explants at $P \leq 0.05$

(I) Genotypes	(J) Varieties	Mean difference (I-J) Shoots number	Std. Error	Sig.	Mean difference (I-J) Shoots length (cm)	Std. Error	Sig.
Florin	Filip	1.1000*	.30687	.001	1.4835*	.20450	.000
	Mimi	1.7500*	.30687	.000	1.0205*	.20450	.000
Filip	Florin	-1.1000*	.30687	.001	-1.4835*	.20450	.000
	Mimi	.6500*	.30687	.039	-.4630*	.20450	.028
Mimi	Florin	-1.7500*	.30687	.000	-1.0205*	.20450	.000
	Filip	-.6500*	.30687	.039	.4630*	.20450	.028

Based on observed means. The error term is mean square (error)=0.942. Shoots number. The error term is mean square (error)=0.418. Shoots lengths (cm). *The mean difference is significant at the 0.05 level.

Table 5. LSD values between variants on shoots number and shoots length per explants at $P \leq 0.05$

(I) Variants	(J) Variants	Mean difference (I-J) Shoots number	Std. Error	Sig.	Mean difference (I-J) Shoots length (cm)	Std. Error	Sig.
V1	V2	-1.8667*	.35434	.000	1.2047*	.23614	.000
	V3	-5.9333*	.35434	.000	.4540	.23614	.060
	V4	-1.2000*	.35434	.001	2.2900*	.23614	.000
V2	V1	1.8667*	.35434	.000	-1.2047*	.23614	.000
	V3	-4.0667*	.35434	.000	-.7507*	.23614	.003
	V4	.6667	.35434	.066	1.0853*	.23614	.000
V3	V1	5.9333*	.35434	.000	-.4540	.23614	.060
	V2	4.0667*	.35434	.000	.7507*	.23614	.003
	V4	4.7333*	.35434	.000	1.8360*	.23614	.000
V4	V1	1.2000*	.35434	.001	-2.2900*	.23614	.000
	V2	-.6667	.35434	.066	-1.0853*	.23614	.000
	V3	-4.7333*	.35434	.000	-1.8360*	.23614	.000

Based on observed means. The error term is mean square (error)=0.942. Shoots number. The error term is mean square (error)=0.418. Shoots lengths (cm). *The mean difference is significant at the 0.05 level.

CONCLUSIONS

The regenerative activity in the studied three varieties depends on the highest level from the genotype, type of the explants and growing season were better regeneration activity is registered with shoot-tip and spring season. The results showed a significant correlation between the concentration of BAP and the shoots number (multiplication rate) and height. The best rate of shoot formation and the highest elongation rate was obtained at a concentration of 5 mg/l BAP (V3).

ACKNOWLEDGEMENTS

The authors thank all colleagues, students, researchers, management and staff in micropropagation laboratory, Centre for Studies of Food and Agricultural Products Quality, USAMV for their assistance in this work.

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