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Original Article

First Report of Krymsk® 5 (cv. VSL 2) Cherry Rootstock *In Vitro* Propagation: Studying the Effect of Cytokinins, Auxins and Endogenous Sugars

Athanasios TSAFOUROS*, Peter A. ROUSSOS

*Agricultural University of Athens, Department of Crop Science, Laboratory of Pomology, Iera Odos 75, Athens 118 55, Greece; thantsaf@hotmail.com (*corresponding author); roussosp@aua.gr*

Abstract

Krymsk® 5 (VSL-2) is a semi-dwarf cherry rootstock adaptable to a range of climates. The present study aimed to establish the first efficient *in vitro* propagation protocol for this rootstock. Therefore, six cytokinines, four adenine type (6-benzyladenine, 2-isopentenyladenine, kinetin and meta-topolin) and two phenylureas (thidiazuron and forchlorfenuron) at three (2.4 μM, 4.8 μM and 9.6 μM) concentrations plus three (0.24 μM, 0.48 μM, 0.96 μM) for thidiazuron only were tested during the multiplication stage. 6-Benzyladenine was the most efficient cytokinin, based on the number of shoots produced (3.5 shoots at 9.6 μM) and the number of nodes per explant (10 nodes at 9.6 μM) whereas the other aromatic adenine tested, i.e. meta-topolin, presented the highest number of nodes per cm and node per shoot. During the rooting stage the synthetic auxins 1-naphthaleneacetic acid (1-NAA) and indolebutyric acid (IBA) were tested at concentrations of 0, 2.5, 5, 10 and 20 μM both separately and in all possible combinations. The percentage of successfully rooted explants reached 95% under the combination of 20 μM IBA plus 5 μM 1-NAA, whereas the highest number of roots recorded was 8.5 roots for the treatment 20 μM IBA plus 2.5 μM 1-NAA. Furthermore, two different carbon sources were compared, the widely used sucrose and the endogenous sugar ratio of mother plant softwood shoot, sampled during late of May. Endogenous sugar ratio proved to be the preferable carbon source, since it increased the number of shoots produced and almost doubled the number of produced nodes per explant.

Keywords: benzyladenine; carbohydrates; furfuryladenine; metaTopolin, micropropagation; *Prunus*

Abbreviations: 1-NAA: 1-Naphthalene acetic acid; 2iP: 2-isopentenyladenine; BA: 6-Benzyladenine; CPPU: Forchlorfenuron; DKW: Driver and Kuniyuki Walnut medium; GA_3: Gibberellic acid; IBA: Indole-3-butyric acid; Kin Kinetin: mTop meta-Topolin; SSR: Sorbitol to Sucrose Ratio; TDZ: Thidiazuron; Z: Zeatin

Introduction

Rootstocks are widely used in agricultural practise in many species (such as plum, peach, pear etc.) for their special properties and characteristics (Webster, 1993). Vigor, yield precocity, adaptation to soil and environmental conditions and resistance to biotic agents are some of the traits evaluated during the development of a new cherry rootstock (Webster, 1993). Krymsk® 5 synonym VSL-2 (*Prunus fruticosa* × *Prunus lannesiana*) is a relatively newly released cherry rootstock. It is characterized as a semi-dwarf rootstock as it reduces trees reaching 85-90% of the size on Mazzard rootstock (Long and Kaiser, 2010). Maas *et al.* (2014) working with cv. 'Kordia' grafted on Krymsk® 5 and GiSelA® 5 rootstocks concluded that fruit production per tree, flowering intensity, shoot growth and trunk diameter was not significantly affected by the rootstock used. Krymsk® 5 is better adapted in heavy soils than 'Mazzard' and is well adapted in both cold and hot climates without leaf cupping as happens to GiSelA® 5 in high temperatures (Long and Kaiser, 2010).

Micropropagation allows the rapid clonal propagation of plant species in limited time and space and is widely used in propagating elite *Prunus* species of an economical and scientific interest including cherry rootstocks and cultivars (Godoy *et al.*, 2017).

Multiple factors affect micropropagation rates, such as the ingredients of a medium and most significantly the plant growth regulators used, as well as their concentration and combinations (George *et al.*, 2008). The major plant growth regulators used in *in vitro* propagation are auxins, mainly during the rooting stage and cytokinins during the multiplication stage (De Klerk *et al.*, 1997; Yancheva and

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Kondakova, 2016). The most widely used cytokines are zeatin (Z), 6-benzylaminopurine (BA), kinetin (Kin), and 2-isopentenyladenine (2iP) (Yancheva and Kondakova, 2016), whereas forchlorfenuron (CPPU) (Caboni *et al*., 2009) as well as thidiazuron (TDZ) have been reported to have cytokinin-like action (Yancheva and Kondakova, 2016). Meta-Topolin (mTop), a natural aromatic cytokinin, has also been used in *in vitro* propagation of various species (Wojtania, 2010; Monticelli *et al*., 2017). Gibberelins (especially gibberelic acid, GA_3) (Gonbad *et al*., 2014) and synthetic auxins (1-naphthaleneacetic acid, 1-NAA or indolebutyric acid, IBA) (Doric *et al*., 2014) have been utilized in combination with BA with satisfactory results during the multiplication stage of some *Rosaceae* and other species and hybrids, resulting in many cases in significantly better results than BA alone (D'Angeli *et al*., 2001; Kadota *et al*., 2001; Andreu and Marın, 2005; Dejampour *et al*., 2011; Sadeghi *et al*., 2015; Akhtar *et al*., 2016).

The effect of the carbon source has been also studied in *in vitro* culture (Nowak *et al*., 2004; Fotopoulos and Sotiropoulos, 2004; Nacheva and Gercheva, 2009) as it plays a crucial role in the successful propagation. Even though sucrose is the most used sugar in *in vitro* cultivation (George *et al*., 2008), other carbohydrates have also been used, resulting in better propagation rates than sucrose (Roussos and Pontikis, 2002). These carbohydrates are in most cases the major carbohydrate of the specific species, justifying partly their superiority in *in vitro* culture. According to Hammatt (1993) the supplementation of growth medium with the carbohydrates found in plant phloem sap could probably be a better carbon source, giving superior results, than the use of sucrose alone.

To our knowledge, this is the first report on the *in vitro* propagation of the cherry rootstock Krymsk® 5. The aim of this study was to establish an efficient *in vitro* propagation protocol by studying the effect of six cytokinines [four adenines (6-benzyladenine, 2-isopentenyladenine, kinetin and meta-topolin) and two phenylureas (thidiazuron and forchlorfenuron)] during the multiplication stage and two auxins (IBA and 1-NAA) during the rooting stage. Furthermore, in order to assess the effect of carbon source on *in vitro* propagation rate, sucrose as well as a combination of endogenously found sugars was also studied.

Materials and Methods

Endogenous carbohydrates analysis

Krymsk® 5 mother plants were maintained at the rootstocks collection of Agricultural University of Athens. Shoot samples from 4 years old trees were collected during late May, a period of fast shoot elongation, transferred to the Laboratory of Pomology under low temperature and placed in a freezer. The samples were lyophilized, ground into fine powder and stored in a freezer (-25 °C) till analysis. For sugar extraction, 50 mg d.w. of sample was extracted with 2.5 ml of HPLC water in a microwave oven at 400 watt for 1.5 min, according to Denaxa *et al*. (2012). The sample was cooled and then centrifuged at 4000 g for 6 min. The supernatant was collected and the pellet was re-extracted with water, following the same procedures. The two supernatants were combined, filtered through a nylon syringe filter (0.45 μm pores) and analyzed by HPLC. The analysis was conducted using a Waters 510 isocratic pump running at 0.6 ml min^{-1} with a Hamilton HC-75 cation exchange column, calcium form (Ca^{2+}) (Hamilton, Bonaduz, Switzerland). The mobile phase consisted of HPLC grade water whereas column was equilibrated at 80 °C. Sugars were detected with an HP 1047A refractive index detector and quantified using a five point calibration curve (y= 25.25x, R^2= 0.998 for sucrose; y= 37.16x, R^2=0.994 for fructose; y=42.157x, R^2=0.998 for glucose, y=48.388x, R^2=0.999 for sorbitol) obtained by known external standards. The endogenous sugar ratio was estimated using at least five shoot samples. Sorbitol:Sucrose ratio (SSR) ratio was estimated as the ratio of endogenous sorbitol and sucrose content.

Explant source and in vitro establishment

Nodal segments (approximately 2 cm) were excised from mother plant softwood shoots collected early in summer. The segments were firstly washed with running tap water to remove any residues. Sterilization comprised the use of 75% v/v ethanol for 13 seconds followed by a period of 15 minutes in 13% v/v sodium hypochlorite low in bromide, plus 2-3 drops of Tween 20. The explants were washed thrice with sterilized deionized water to remove any traces of the disinfectant.

After sterilization, the explants were planted in test tubes containing 10 ml of Driver and Kuniyuki Walnut medium (DKW), supplemented with 9 g L^{-1} Agar, 30 g L^{-1} sucrose, 4.8 μM BA, 0.7 μM GA_3 and 0.5 μM 1-NAA. The pH was adjusted at 5.8 (prior autoclaving). After a period of four weeks the produced shoots were excised and transplanted for eight weeks, in a same, fresh substrate in order to increase the number of explants. All explants were cultured under 22 ± 1 °C, 16h of photoperiod and light intensity of 3000 lux provided by cool daylight fluorescent lamps.

Effect of cytokinin type and carbon source on explant multiplication rate

In order to assess the effect of carbon source on *in vitro* performance of Krymsk® 5 explants, microshoots of approximately 1 cm were transplanted in DKW substrate supplemented with 4.8 μM BA, 0.7 μM GA_3 and 0.5 μM 1-NAA, containing either a) sucrose (30 g L^{-1}) as source of carbon, which served as control treatment, or b) a mixture of endogenously found carbohydrates at a ratio of 2.4 g L^{-1} sucrose, 2.4 g L^{-1} fructose, 5.2 g L^{-1}, glucose and 20 g L^{-1} sorbitol. The explants remained at these media for eight weeks, after which the number of shoots, their length and the number of nodes were measured.

A second experiment took place, in order to assess the efficacy of six different cytokinins on *in vitro* multiplication rate. Treatments consisted of three concentrations of each cytokinin, except from TDZ where six concentrations were assessed. More specifically, the following cytokinins BA, Kin, 2iP, mTop, CPPU and TDZ were applied at concentrations of 2.4, 4.8 and 9.6 μM, whereas TDZ was also tested at lower concentrations i.e. 0.24, 0.48 and 0.96.

mTop and CPPU were filter sterilized, while the rest of cytokinins were autoclaved. The substrate was supplied with the endogenous sugar ratio as indicated by the HPLC analysis. After a period of eight weeks the mean shoot length, the number of produced shoots and the number of nodes were measured.

Effect of exogenously applied auxins in in vitro rooting performance of Krymsk® 5 rootstock

For the rooting experiments, *in vitro* produced explants, approximately 1.5 cm in length, were transferred to the rooting substrate (DKW medium) supplemented with 20 g L^{-1} sucrose and 9 g L^{-1} agar). The two commonly used synthetic auxins IBA and 1-NAA were used at concentrations of 0, 2.5, 5, 10 and 20 μM both separately and in all possible combinations. In total, 25 rooting treatments took place.

Statistical analysis

The experiment was arranged according to the completely randomized design (CRD) with four replications of five explants. The raw data were analyzed by analysis of variance (ANOVA) whereas, principal component analysis (PCA) was used in order to visualize data variance. Statistical analysis was carried out using JMP 10.0 (SAS, U.S.A.) and Statgraphics Centurion XV software for PCA and ANOVA analyses respectively. Each experiment was repeated twice. Statistically significant differences among means were detected using the LSD test at $p \leq 0.05$.

Results and Discussion

HPLC analysis and endogenous sugar ratio

Krymsk® 5 is a cherry rootstock of the *Rosaceae* family. Sorbitol was found to be the dominant sugar in softwood shoots of this cherry rootstock during late May as can be seen in Table 1. This is in agreement with the literature where sorbitol is reported to be the main sugar in *Rosaceae* species (Marino *et al*., 1993; Moing *et al*., 1997; Bianco and Rieger 2002; Yaseen *et al*., 2013). As far as the other sugars are concerned, fructose and sucrose have been found to participate equally, whereas glucose is the second most abundant sugar. Similar findings, had been reported by Ranney *et al*. (1991) working with 'Colt' cherry rootstock and sour cherry cv. 'Meteor'.

The Sorbitol:Sucrose ratio (SSR) has been related to environmental conditions where the plant is growing (Moing *et al*., 1997; Kanayama 2009). According to Moing *et al*. (1997) in mature leaves this ratio ranges from approximately 0.81 to 5.59. In the present study, the ratio exceeds that found in mature leaves, probably due to different genotype studied and geographical origin as Moing *et al*. (1997) suggested.

Effect of the sugar source on shoot proliferation

Sugars can affect plant development *in vitro,* influencing plant physiology and morphogenesis. Supplementing the growth medium with the carbohydrates found endogenously in actively growing shoots resulted in insignificantly higher number of shoots and nodes produced per explant in comparison to sucrose (Table 2). Sucrose has been found to be the main sugar in the phloem sap of many species (Fuentes *et al*., 2000; Ahmad *et al*., 2007) and is used as the main carbon source in micropropagation due to its low price and high efficiency (George *et al*., 2008). On the other hand, Hammatt (1993) suggested that better results can be achieved when the proportions of the phloem sap sugars are used *in vitro* supported by the present results (Table 2).

Based on the above results, a substrate containing the endogenous sugar ratio, characterized by high concentration of sorbitol, is more efficient during the proliferation stage of Krymsk® 5 instead of an exclusively containing sucrose substrate. Sorbitol efficiency on *Rosaceae* family species micropropagation has been long known. Sorbitol included in the substrate was superior to sucrose in pear (Kadota *et al*., 2001), apricot (Marino *et al*., 1993), apple (Moncousin *et al*., 1992), peach rootstock GF677 (Ahmad *et al*., 2007) and others, increasing in some species the number of microshoots produced (Borkowska and Szczerba, 1991; Marino *et al*., 1993), or shoot elongation (Marino *et al*., 1993). Furthermore, in the *in vitro* multiplication stage of 'Lapins' sweet cherry and 'Tabel Edabriz' cherry rootstock, sucrose was the least efficient carbon source while sorbitol, glucose and fructose gave better results (Ruzic *et al*., 2008a). On the other hand, Cheong and An (2015) studying eight different *Prunus* species, concluded that only for *Prunus salicina* and *Prunus tomentosa* fructose and glucose enhanced adventitious shoot induction compared to sucrose, but sorbitol was not included in the experiment. Although sorbitol seems to be the preferable sugar for some *Rosaceae* species in *in vitro* proliferation, a mixture of sugars based on the endogenously detected shoot sugar proportion has not be tested yet (according to our knowledge). Thus, the effects of the different carbon sources on explant growing during *in vitro* proliferation are not sufficiently studied.

Table 1. Endogenous sugar percentage in Krymsk® 5 shoots during late May

	Sugars				
	Sucrose	Fructose	Glucose	Sorbitol	SSR[1]
Percentage (%)	8	8	17	67	8.3

[1]SSR: Sorbitol to Sucrose ratio

Table 2. Effect of sugar source on shoot proliferation variables

Source of carbon	Shoots per explant	Shoot length (cm)	Nodes per explant	Nodes per shoot	Nodes per cm
Endogenous mix	2.06 b	1.28 a	9.45 b	4.31 a	3.45 a
Sucrose	1.29 a	1.23 a	5.52 a	3.85 a	3.22 a

Means within the same column followed by the same letter do not differ significantly according to the LSD multiple range test ($p \leq 0.05$)

Borkowska and Szczerba (1991) using a sugar mixture comprising of sucrose, glucose and fructose in equal proportions concluded that the proliferation rate was the lowest among treatments and that generally the presence of fructose in the medium decreased the number of shoots. On the contrary, Cheong and An (2015) and Rugini *et al.* (1987) reported that fructose can improve proliferation parameters in some species.

In the present study, the presence of fructose did not have the negative impact described by Borkowska and Szczerba (1991) indicating that both the type of sugar and the applied concentration of each sugar as well as the interactions among them can have a significant effect. Although Borkowska and Szczerba (1991) agree that fructose presence in the medium can increase shoot length of sour cherry explants, this is not confirmed by the present results, probably due to either different genotype and/or presence of other carbohydrates in higher concentrations than fructose.

Effect of cytokinin treatments on shoot proliferation

Many times the supplementation of a growth medium with only cytokinins is not sufficient to induce the formation of axillary shoots. This was observed in preliminary experiments with Krymsk® 5 explants grown in DKW medium supplied only with BA (data not shown). The simultaneous presence of cytokinin, giberellin and auxin, in the nutrient substrate has proven to be more efficient for *Prunus* rootstocks *in vitro* according to Brison *et al*. (1995).

When the main effects of cytokinins were tested irrespective of their concentration it was found that all used adenines resulted in statistically higher shoot length than phenylureas (Table 3). Among the cytokinins tested, BA and CPPU produced the highest number of shoots per explant, resulting in 2.23 and 1.87 shoots respectively. CPPU has been found to be effective in adventitious shoot regeneration of *Actinidia deliciousa* cv. 'Tomuri' and 'Hayward', mulberry, avocado and raspberry (Caboni *et al*., 2009) and adventitious shoots formation from callus of *Malus pumila* (Kadota and Niim, 2003). On the other hand, BA is maybe the most used and effective cytokinin in *Prunus* micropropagation (Borkowska and Szczerba, 1991; Marino *et al*., 1993; Rossi *et al*., 1993; Nowak *et al*., 2004; Cheong and An, 2015; Wiszniewska *et al*., 2016) and is suggested as the preferable cytokinin for many *Prunus* rootstocks such as 'GF677' (*Prunus amygdalus* × *Prunus persica*) (Alanagh *et al*., 2010), Adesoto (*Prunus insititia*) (Andreu and Marın, 2005), 'HS 314' (*Prunus amygdalus* × *Prunus persica*) (Dejampour *et al*., 2011), 'Fereley-Jaspi' (*Prunus salicina* × *Prunus spinosa*), 'Ferlenain-plumina' (*Prunus besseyi* × unknown parent) (Brison *et al*., 1995) etc. mTop has been found to be better than BA in *Musa* spp. (Escalona *et al*., 2003) and *Pelargonium* (Wojtania, 2010). Gentile *et al*., (2014) working with the *Prunus* rootstocks 'Ferdor' and 'Torinel' and Monticelli *et al*. (2017) working with 'Penta' and 'Myrobalan 29C' found out that mTop did not improve any further the proliferation rate compared to BA as has been found in the present experiment too. In the present study, Kin and 2iP were generally ineffective compared to BA for all the parameters measured. The same tendency has been observed in many *Prunus* species using Kin and 2iP and it might be the explanation why 2iP and Kin are not usually used in micropropagation of fruit species (Ruzic and Vujovic, 2008b). On the other hand, Kin has been reported to be the most suitable among BA, TDZ and Z for shoot multiplication of cucumber (Abu-Romman *et al*., 2015) indicating that the suitable cytokinin type for *in vitro* propagation is genotype dependent. Despite the fact that TDZ produced better results in apple shoot proliferation than adenines (Kadota *et al*., 2003), in the present trial it presented the lowest efficacy, indicating that Krymsk® 5 prefers adenine type cytokinin compared to phenylureas.

With the aim of enabling a better and simple visual interpretation of adenine efficacy, a PCA was conducted (Fig. 1). PCA revealed that adenine cytokinins could be separated into two groups. Group I consisted of BA and characterized by high number of nodes per explant and shoot, while the rest of adenines (Kin, mTop and 2iP) comprised the second group (Group II) characterized by high shoot length. The first component of the analysis explained 38.5% of the variation and the second one an additional 33.1% that is a total of 71.6% (Fig. 1). The first component was associated with shoot number, whereas the second one with shoot length.

Medium autoclaving has no significant impact on the physical stability of all of the four adenine cytokinins assayed (Hart *et al*., 2016). Therefore any difference in the effectiveness among cytokinins could be partly attributed to the different metabolism of them within the plant. BA higher efficacy could be assigned to the fact that it is not a suitable substrate of cytokinin oxidase (van Staden and Crouch, 1996; Kieber and Schaller, 2004; George *et al*., 2008), the enzyme responsible for the endogenous cytokinin homeostasis (Schmülling *et al*., 2003).

Table 3. Effect of cytokinins on proliferation parameters

Parameters	Cytokinin					
	Adenines				Phenylureas	
	BA	2iP	mTop	Kin	TDZ	CPPU
Shoots per explant	2.23 c	0.72 ab	0.98 b	0.57 a	0.4 a	1.87 c
Shoot length (cm)	1.16 b	1.49 c	1.41 bc	1.37 bc	0.56 a	0.5 a
Nodes per explant	8.4 e	3.95 cd	4.9 d	2.8 ab	2.3 a	3.5 bc
Nodes per shoot	3.88 c	3.9 c	4.45 c	2.85 b	2.13 ab	1.86 a
Nodes per cm	3.45 bcd	2.7 ab	3.84 cd	2.16 a	2.84 abc	4.19 d

Means within the same row followed by the same letter do not differ significantly according to LSD multiple range test at $p \leq 0.05$

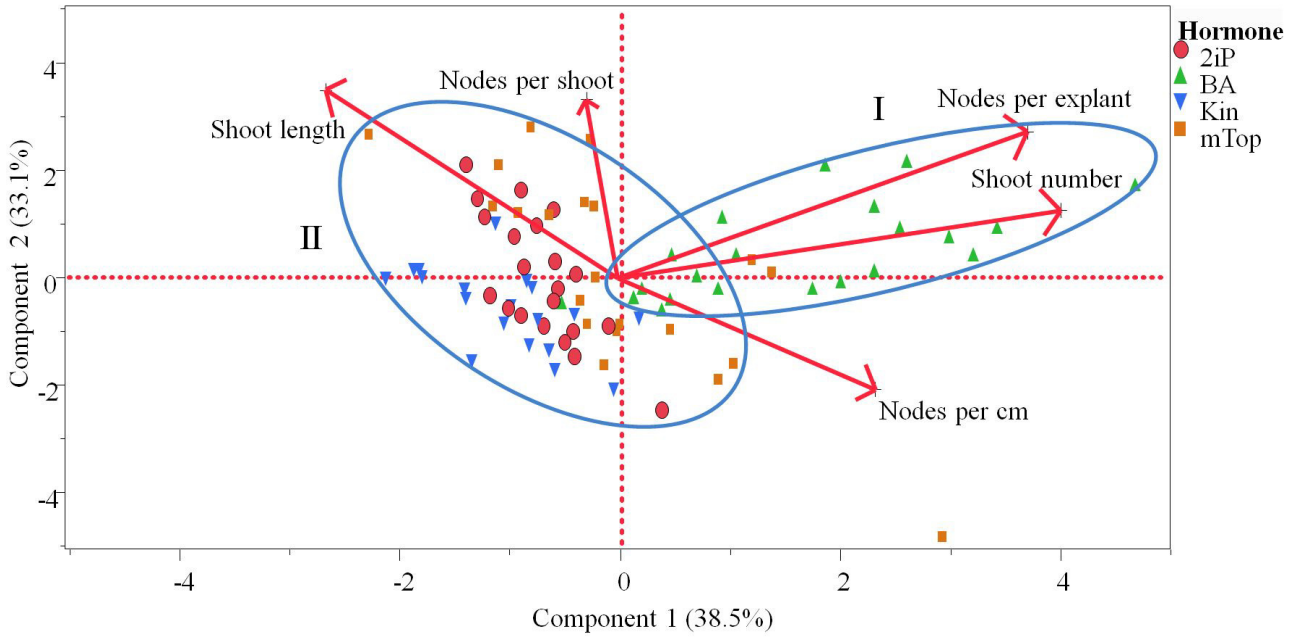

Fig. 1. Biplot of the proliferation parameters (shoot length, nodes per shoot, shoot number produced per explant, node number per explant and nodes per cm) of shoots produced after adenine treatments [BA(triangle marker), Kin (reverse triangle marker), mTop (square marker) and 2iP (cycle marker)] on Krymsk® 5 explants and adenine grouping (groups I and II).

Furthermore, some of the conjugated forms of BA produced during BA metabolism, present cytokinin activity (van Staden and Crouch, 1996; Zhang *et al*., 2010), extending thus its action. Moreover, BA can be characterized as the most stable among the cytokinins tested, since the stability of the aromatic side chain substituted at N^6 is higher than the isoprenoid chain of 2iP, the furan chain of Kin and the phenol chain of mTop.

When the effect of each cytokinin in equimolar concentration was tested, it was evident that the maximum number of shoots per explant was achieved by the use of 9.6 μM BA (3.6 shoots) (Fig. 2). Increasing the concentration of all three BA, mTop and TDZ resulted in increased number of shoots per explant. Higher concentrations of BA have been used in *in vitro* culture of other *Prunus* species, indicating that a higher concentration of BA i.e. higher than 9.6 μM could be effective for Krymsk® 5 too (Tiwari *et al*., 2001; Kodota *et al*., 2003; Alanagh *et al*., 2010; Gonbad *et al*., 2014; Fallahpour *et al*., 2016).

As far as the shoot length is concerned, adenines produced longer shoots than phenylureas, as indicated in Fig. 3. Kin, 2iP and mTop addition in the medium resulted in high shoot length, followed by BA. Similar results have been reported by other researchers working with either cherry cultivars or apricot ones (Murai *et al*., 1997; Ruzic and Vujovic, 2008b), while according to Fallahpour *et al*. (2016) in GiSelA® 5 rootstock the highest shoot length can be obtained by the simultaneous addition of BA and Kin in the medium. CPPU as well as TDZ resulted in decreased shoot length, irrespective of the concentration used, in agreement with Huetteman and Preece (1993) and Pruski *et al*. (2005) who found that TDZ inhibited shoot elongation and retarded shoot growth in comparison to BA, Kin and 2iP. Thus, it seems that phenylureas exhibit an inhibitory effect on shoot growth of Krymsk® 5 rootstock.

As node constitutes the propagation unit for *in vitro* micropropagation it is important for a micropropagtaion protocol to achieve the highest number of nodes per explant, during the proliferation stage. The results suggested the 4.8 μM and 9.6 μM of BA were the treatments with the highest node production per explant (Fig. 4). The higher the concentration of BA used the higher was the number of nodes, especially between the low and medium BA concentration. The two highest BA concentrations resulted in the highest number of shoots per explant (Fig. 4), indicating that the increased node production is strongly related to the number of shoots of this rootstock (Fig. 1).

The maximum node number per shoot was achieved by 9.6 μM mTop (Fig. 5). A tendency of increased number of nodes per shoot per increased cytokinin concentration was recorded under all three 2iP, TDZ and mTop addition, while the opposite stood for CPPU addition. This indicates that all three previous mentioned cytokinins are effective in inducing high number of nodes per shoot, but are less effective on inducing axillary shoot formation (Fig. 2). One could suggest that a combined effect of BA on inducing high number of shoots along with 2iP and/or TDZ and/or mTop could result in the maximum production of nodes per explants, exploiting at the same time the efficiency of BA on increasing shoot production and the efficiency of the other three cytokinins on increasing node formation per shoot.

On the other hand, the node number per shoot cm, which represents the density of nodes on the shoot and indirectly the length of internode was highest under 2.4 μM of mTop (Fig. 6). TDZ followed the same pattern as previously, increasing the node density as the concentration elevated (Fig. 6). The lowest density of nodes was achieved under Kin inclusion the medium, indicating that the specific cytokinin affects internode length, producing

explants with larger internodes and easy to handle propagules.

Effect of different TDZ concentrations

TDZ has been used successfully in the *in vitro* propagation of various plant species stimulating shoot formation but in lower concentrations compared to adenines (Huetteman and Preece, 1993). When used in high concentrations, i.e. concentrations used for the adenine cytokinins, TDZ may cause a series of undesirable reactions such as somatic embryos development, callus formation, shoot growth inhibition (Huetteman and Preece, 1993; Guo *et al*., 2011), shoot hyperhydricity and fasciation (Guo *et al*., 2011). According to Huetteman and Preece (1993) in order to define the best TDZ concentration, preliminary experiments should be conducted with equimolar concentrations between adenines and TDZ as well as lower level concentrations of TDZ. Since TDZ in the present experiment was the least effective cytokinin for the *in vitro* propagation of Krymsk® 5, lower concentrations were also used, in order to assess their effects. Thus, TDZ was also tested at 1/10th of the concentrations initially used, ranging from 0.24 μM to 9.6 μM (Table 4). There were not any significant differences for the parameters measured except for shoot length, where the 0.48 μM treatment resulted in the maximum length. In general, lower concentrations resulted in longer shoots confirming that high TDZ concentration may inhibit shoot growth (Huetteman and Preece, 1993; Guo *et al*., 2011). Callogenesis was also observed but there were not significant differences among the different concentrations (data not shown). It seems that Krymsk® 5 responds better to the presence of adenine

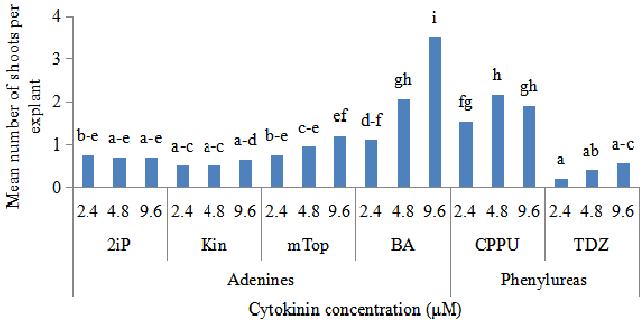

Fig. 2. Number of axillary shoots produced per explant after two months of cultivation in six different cytokinins (2iP, Kin, mTop, BA, CPPU and TDZ) and three cytokinin concentrations (2.4 μM, 4.8 μM and 9.6 μM). Columns followed by the same letter do not differ significantly according to the LSD multiple range test (p≤0.05)

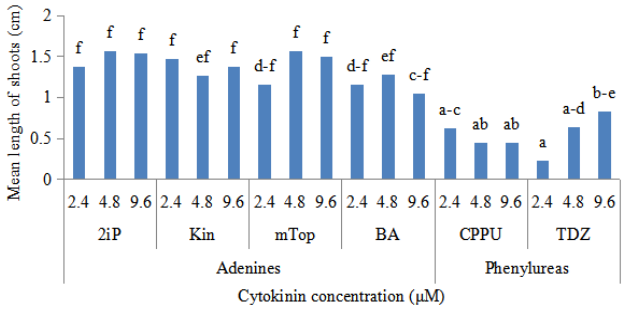

Fig. 3. Average length (in cm) of axillary shoots produced per explant after two months of cultivation in six different cytokinins (2iP, Kin, mTop, BA, CPPU and TDZ) and three cytokinin concentrations (2.4 μM, 4.8 μM and 9.6 μM). Columns followed by the same letter do not differ significantly according to the LSD multiple range test (p≤0.05)

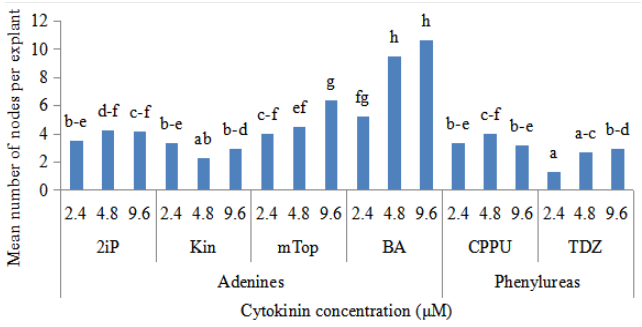

Fig. 4. Average number of nodes produced per explant after two months of cultivation in six different cytokinins (2iP, Kin, mTop, BA, CPPU and TDZ) and three cytokinin concentrations (2.4 μM, 4.8 μM and 9.6 μM). Columns followed by the same letter do not differ significantly according to the LSD multiple range test (p≤0.05)

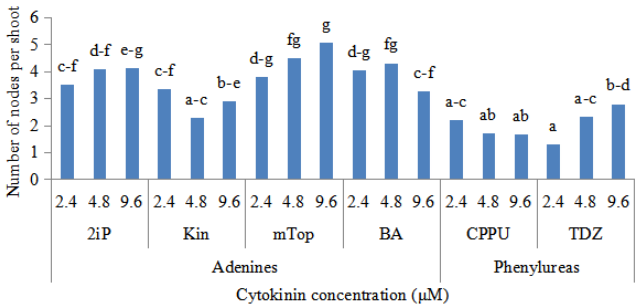

Fig. 5. Average number of nodes per shoot produced after two months of cultivation in six different cytokinins (2iP, Kin, mTop, BA, CPPU and TDZ) and three cytokinin concentrations (2.4 μM, 4.8 μM and 9.6 μM). Columns followed by the same letter do not differ significantly according to the LSD multiple range test (p≤0.05)

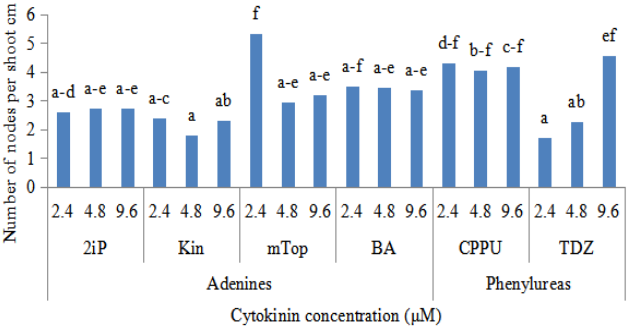

Fig. 6. Average number of nodes per cm of shoot produced after two months of cultivation in six different cytokinins (2iP, Kin, mTop, BA, CPPU and TDZ) and three cytokinin concentrations (2.4 μM, 4.8 μM and 9.6 μM). Columns followed by the same letter do not differ significantly according to the LSD multiple range test (p≤0.05)

Table 4. Effect of different TDZ concentrations on proliferation variables

Parameters	TDZ (μM)					
	0.24	0.48	0.96	2.4	4.8	9.6
Shoots per explant	0.64 a	0.64 a	0.57 a	0.22 a	0.41 a	0.57 a
Shoot length (cm)	1.11 bc	1.54 c	0.87 a-c	0.23 a	0.63 ab	0.83 a-c
Nodes per explant	3.00 a	3.21 a	2.74 a	1.29 a	2.67 a	2.9 a
Nodes per shoot	3.00 a	3.21 a	2.74 a	1.29 a	2.31 a	2.80 a
Nodes per cm	2.48 a	1.95 a	4.5 a	1.7 a	2.25 a	4.56 a

Means within the same row followed by the same letter do not differ significantly according to the LSD multiple range test (p≤0.05)

Table 5. Main effects of auxin type and their interaction on rooting variables

Auxin	Rooted explants (%)	Number of roots	Length of roots (cm)
Control	40 a	6.8 ab	0.24 a
IBA	55 a	4.55 a	0.32 a
1-NAA	80.4 b	6.15 ab	0.35 a
IBA*1-NAA	70.3 b	7.15 b	0.31 a

Means within the same column followed by the same letter do not differ significantly according to the LSD multiple range test (p≤0.05)

Table 6. Effect of auxin combinations on rooting variables

Auxin (Concentration (μM))		Rooted explants (%)	Number of roots	Length of roots (cm)
IBA	NAA			
0	0	40 ab	6.8 b-f	0.23 a-c
0	2.5	90 fg	5.9 a-f	0.44 d-g
0	5	67 b-g	3.3 a	0.19 a
0	10	90 fg	8.2 f	0.55 fg
0	20	75 d-g	7.1 c-f	0.22 ab
2.5	0	50 a-d	4.9 a-e	0.23 a-c
5	0	45 a-c	3.6 ab	0.61 g
10	0	57 a-e	4.8 a-d	0.16 a
20	0	68 b-g	4.9 a-e	0.28 a-d
2.5	2.5	32 a	5.3 a-f	0.19 a
5	5	43 ab	4.4 a-c	0.15 a
10	10	85 e-g	7.6 c-f	0.18 a
20	20	85 e-g	7.6 c-f	0.18 a
2.5	5	83 e-g	7.5 c-f	0.47 d-g
2.5	10	67 b-g	5.7 a-f	0.27 a-d
2.5	20	73 c-g	7.6 c-f	0.22 ab
5	2.5	50 a-d	8.3 f	0.40 b-f
5	10	85 e-g	7.3 c-f	0.23 a-c
5	20	82 e-g	8.0 d-f	0.29 a-d
10	2.5	63 b-f	6.8 b-f	0.32 a-e
10	5	58 a-d	6.1 a-f	0.22 ab
10	20	63 b-f	8.1 ef	0.40 b-f
20	2.5	75 d-g	8.5 f	0.48 d-g
20	5	95 g	8.2 f	0.46 d-g
20	10	85 e-g	7.3 c-f	0.30 a-d

Means within the same column followed by the same letter do not differ significantly according to the LSD multiple range test (p≤0.05)

cytokinins in the medium than phenylureas, which has been observed in other species too (Murai *et al*., 1997; Ružić and Vujović, 2008; Rafique and Anis, 2014).

Effect of auxin treatments on rooting

From the analysis of auxin type main effects it was concluded that the highest rooting percentage was achieved by the addition of 1-NAA the medium, reaching 80% (Table 5). IBA inclusion did not induce a high rooting percentage, as this was similar to that achieved under control condition (no auxin in the medium). Similar results have been found by Tang *et al*., (2001) who concluded that NAA treated explants of 'Napoleon' and 'Beutel Spacher Rexelle' cultivars rooted in higher percentage in than the IBA treated ones. The combination of the two auxins resulted also in high rooting percentages.

Thus, it seems that Krymsk® 5 responds better to the presence of 1-NAA in the medium, despite the fact that for

many *Prunus* species IBA has been used as the preferable auxin for rooting induction (Kalinia and Brown 2007; Dejampour *et al*., 2011; Sadeghi *et al*., 2015) while sometimes 1-NAA has been found to be ineffective on inducing rooting (Dejampour *et al*., 2011). Working on micropropagation of olive, Canas (1988) concluded that IBA is more efficient for some cultivars whereas 1-NAA for others. It is clear then that the genotype greatly influences explant response to the presence of a specific auxin and could explain our findings with Krymsk® 5 compared to those in other *Prunus* genotypes. This is further corroborated by Dolcet-Sanjuan *et al*. (2004) working with walnut, who reported that rooting response depends on many factors, among which the type of auxin used, the applied concentration, the species or even the clone of a specific species.

The number of roots is a significant factor for increased plant survival percentage during the acclimatization phase and it is considered as a qualitative trait of rooting response (Roussos *et al*., 1999; Dolcet-Sanjuan *et al*., 2004). The combination of both IBA and 1-NAA irrespective of the concentrations used, resulted in the highest number of roots while IBA alone exhibited significantly fewer roots (Table 5).

The highest root induction was achieved when 20 μM of IBA and 5 μM of 1-NAA were combined, reaching 95% of rooted explants, followed by 1-NAA alone at 2.5 and 10 μM (90%) (Table 6). Similar results, under IBA and NAA combination were achieved by Mangal *et al*. (2014) in *in vitro* rooting of olive cv. 'Frantoio' and by Roussos *et al*. (1999) working with jojoba. As far the root number is concerned, the highest number was obtained by the combinations 5 μM IBA - 2.5 μM NAA, 20 μM IBA - 2.5 μM NAA, 20 μM IBA – 5 μM NAA, 20 μM IBA – 10 μM NAA and 10 μM NAA, indicating that IBA and NAA may act synergistically on root elongation. Increasing IBA concentration did not increase root number, unlike the results reported by Fotopoulos and Sotiropoulos, (2005) and Fallahpour *et al*. (2015) in PR 204/84 and GiSelA® 5 respectively. The highest root length was observed under IBA at 5 μM and the lowest under the equimolar addition of both auxins at 5 μM. Hossini *et al*. (2010) working on GiSelA® 6 reported that increased IBA concentration caused a significant shortening of roots similar to that observed in the present trial. During the acclimatization stage under mist approximately 90% of rooted explants survived and continued growing.

Conclusions

The first protocol for efficient *in vitro* propagation of the cherry rootstock Krymsk® 5 was established in the present study. During the proliferation stage the DKW nutrient medium, adjusted at pH 5.8, supplied with 30 g L^{-1} of sugars (2.4 g L^{-1} sucrose, 2.4 g L^{-1} fructose, 5.2 g L^{-1}, glucose and 20 g L^{-1} sorbitol), 9 g L^{-1} agar, 9.6 μM BA, 0.7 μM GA_3 and 0.5 μM NAA resulted in a high proliferation rate. During the rooting stage, the DKW medium adjusted to pH 5.8 and supplied with 20 g L^{-1} sucrose, 9 g L^{-1} agar and an auxin combination of 5 μM IBA and 20 μM NAA resulted in the optimum rooting response. The present findings can be successfully utilized in both commercial and experimental large scale clonal *in vitro* propagation of this rootstock.

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