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PLANT TISSUE CULTURE

Optimizing micropropagation conditions for a recalcitrant ninebark (*Physocarpus opulifolius* L. maxim.) cultivar

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Abstract

Ninebark is a very popular ornamental shrub. Micropropagation is an efficient method for mass production of uniform plant material. This study was designed to develop and optimize conditions at all phases of ninebark micropropagation. For the multiplication stage, the Murashige and Skoog (MS) medium at full concentration and pH 5.8 was chosen as the basal medium. Sorbitol proved a more effective carbohydrate source than fructose, with no adverse effects on shoot vitrification or the medium itself. The best shoot production, both in number and length, was on the medium enriched with 2 and 3 mg·L^{-1} zeatin. High numbers of shoots were also obtained in treatments with 1 mg·L^{-1} 6-benzyladenine (BA) or 2 mg·L^{-1} meta-Topolin (mT) in the basal medium. BA was the most cost-effective cytokinin. There was a positive effect of the gibberellic acid on proliferation: the highest shoot number per explant was produced in the presence of 1 mg·L^{-1} GA_3. No effect of the culture age (up to 20 subcultures) on the percentage of regenerating explants was evident, and the highest numbers of shoots were obtained between passages 10 and 17. For rooting, the MS medium at half strength was used. The best rooting was at 1 mg·L^{-1} IBA. Spraying the *in vitro* rooted cuttings with abscisic acid (ABA) favored plant acclimation to the ex *vitro* conditions. *Ex vitro* rooting, including the treatments with IBA and ABA, shortened the production time by approximately one third.

Keywords Auxin · Cytokinin · Carbohydrate source · *In vitro* · Plant growth regulators

Introduction

Ornamental shrubs, also used in reforestation programs and soil retention systems, are in high demand in private and urban green areas. To meet the growing demand the nurserymen seek new and more efficient propagation. As a rule, conventional propagation techniques are not sufficiently effective in propagation of woody plants and require large quantities of stock plants.

Ninebark has become incredibly famous recently. Its several cultivars are hardy, colorful, and well-growing in full sun or semi-sunshine tolerating a wide range of soil types. Because the leaves are insensitive to air pollution, it is also used for phytoremediation (Popek *et al.* 2018). However, there are general problems in conventional propagation of ninebark as the rooting rate of stem cuttings differs among the cultivars (50–85%), at times falling below the economic threshold, i.e., 50% (Pacholczak and Szydło 2008). Without a rooting stimulator, the cultivar used here rooted only in 26% when propagated by semi-lignified cuttings (Pacholczak 2015). Thus the *in vitro* micropropagation appears as an effective alternative to the standard propagation by cuttings as—according to literature—it is capable for producing large quantities of the true-to-type plant material (Hussain *et al.* 2012). The latter aspect is crucial in cultivar propagation as they do not breed true when propagated by seeds.

The *in vitro* shoot **multiplication** and **rooting** are affected by several factors (Haque *et al.* 2003), one of which is the concentration and type of the exogenous carbon sources in the medium as an energy source and the osmotic potential regulator (De Neto and Otoni 2003). A continuous supply of carbohydrates from the medium is necessary for plants cultured *in vitro*, because under low light intensity, limited gas exchange and high relative humidity in growth chambers, the photosynthetic activity is greatly reduced (Kozai 1991). Earlier trials on ninebark identified fructose as the best carbon source for multiplication and rooting of the cultivar DIABLE D'OR 'Mindia' (Ilczuk *et al.* 2013). However, vitrification of

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new axillary shoots produced on the MS medium supplemented with fructose was frequent, and issues with media solidification were frequent (unpublished data). In this study, fructose was compared with another carbohydrate, sorbitol. Sorbitol is said to be a suitable carbon source for plants of *Rosacae* family where ninebark belongs (Moing *et al.* 1992; Ahmad *et al.* 2007; Yaseen *et al.* 2013). Perhaps positive effects of sorbitol can be attributed to the fact that it is the main photosynthesis product in this family (Moing *et al.* 1992).

Nutrient composition of a culture medium is critical for the initial explant response (Hussain *et al.* 2012), and different media with different formulations are in use. In 1962, Murashige and Skoog developed the nutrient composition (MS) with high amounts of potassium and nitrogen ions. Although originally developed for tobacco (*Nicotiana*), it has become the most popular medium for the tissue culture in a wide range of plants. On the other hand, the formulation of Nitsch and Nitsch in 1969 (NN) has less than half the amount of salts as compared with MS; in Schenk and Hildebrandt's (1972) medium developed for suspension cultures, a high proportion of potassium nitrate is present. The medium of Quoirin an Lepoivre (1977) formulated for propagation of *Prunus*, contains one fourth of NH_4NO_3 present in the MS and is supplemented with $Ca(NO_3)_2$. For *Rubus*, Anderson (1980) developed the medium containing one fourth of the amounts of NH_4NO_3 and KNO_3 present in MS but also containing $NaH_2PO_4{\cdot}H_2O$. In 1980 Lloyd and McCown prepared a medium for micropropagation of woody plants (WPM), more diluted than MS.

Plant growth regulators (PGRs) affect cell divisions and their differentiation as well as further plant growth. Therefore, their presence in a culture medium is crucial. The type and concentration of a PGR depends on the plant species and the explant type as well as the experimental goal. Generally, high auxin concentrations favor root development, while high concentrations of cytokinins stimulate shoot regeneration (Perveen *et al.* 2011; Hussain *et al.* 2012).

Cytokinins are adenine derivatives capable of inducing cell divisions in tissue cultures, initiating shoots and stimulating their proliferation and further growth, both of which are critical for *in vitro* multiplication efficiency. Exogenously applied cytokinins are more active in the presence of an auxin (Perveen *et al.* 2011; Faisal *et al.* 2018; Nowakowska *et al.* 2019). Frequently used cytokinins are of natural origin such as zeatin (Zea), the 2-isopenthenyladenine (2iP), kinetin (Kin), and 6-benzyladenine (BA) or synthetic cytokinins such as thidiazuron (TDZ) (Pierik 1997). BA is the most commonly used cytokinin for micropropagation, but plant taxa differ in their preference towards specific cytokinins. Recently, meta-Topolin (mT) has become a more common cytokinin in the *in vitro* plant production (Werbrouck *et al.* 1996; Gentile *et al.* 2014; Koszeghi *et al.* 2014). In certain species gibberellic acid (GA_3) may also induce the formation of axillary shoots (George *et al.* 2007). GA_3 in combination with BA accelerated *in vitro* shoot multiplication in certain wild roses, thus increasing their micropropagation efficiency (Pawłowska 2011).

An important factor during plant micropropagation is the number of subcultures (passages). The effect of subculture on the multiplication rate varies from one species to another, optimization of the number of passages is a very important aspect of the entire protocol (Kumar *et al.* 2010). Repeated subculture cycles can reduce the plant regenerative capacity as in *Gmelina arborea* (Naik *et al.* 2003) or in fruit tree root stocks (Vujović *et al.* 2012). A drop in the multiplication rate in consecutive passages of shoots on medium with a constant hormonal composition was also observed in six species and cultivars of *Rosaceae* (Norton and Norton 1986). However, in *Mahonia leschenaultia*, repeated passages of shoot cultures through at least 10 cycles resulted in successful proliferation without a loss of vigor, growth, or morphological abnormalities (Radha *et al.* 2013). Hence, the effect of the long-term growth in the same culture medium (20 subcultures, i.e., over 3 years) was also an important aspect of this study.

Rooting of cuttings is a critical step in micropropagation of woody plants where adventitious root development is more difficult than in herbaceous species. Inability to induce adventitious roots may be a barrier to use *in vitro* cultures to propagate trees and shrubs (Yan *et al.* 2010; Shekhawat and Manokari 2016). The main plant growth regulator responsible for rooting is **auxin.** Auxin as such does not participate in the formation of root primordia but activates the already existing ones or induces the new ones (De Klerk *et al.* 1999). For rooting of microcuttings, the indole-3-butyric acid (IBA) is mostly used (Ďurkovič and Bukovská 2009; Feng *et al.* 2009), while 1-naphtalene-acetic acid (NAA) and indole-3-acetic acid (IAA) are less frequently applied (Ďurkovič 2008; Ďurkovič and Bukovská 2009; Hartmann *et al.* 2011).

When transferred to the *ex vitro* conditions, plants may be damaged by rapid changes in the environmental conditions. This calls for a period of **acclimation** as the final phase of micropropagation. Micropropagated plants are produced under high humidity; therefore, they often have poorly developed epicuticular waxes and the cuticle, their stomata function incorrectly so their photosynthesis rate is low (Hazarika 2006; Mišalová *et al.* 2009). As stomata do not close properly, the transpiration rate is high, and this may severely impact the survival rate of microcuttings when they are transferred to the *ex vitro* conditions (Ziv 1995). The process of acclimation can be accelerated by hardening plants *in vitro* or after planting them out in a greenhouse. The transpiration rate should be limited under such conditions, for example, by antitranspirants such as the abscisic acid (ABA) (Pospisilova *et al.* 1999). ABA increases plant tolerance to environmental stresses—hastens stomata closing thus limiting water losses (Rai *et al.* 2011). Light is another important environmental

factor which should be manipulated during plant acclimation. An increase in its intensity stimulates growth of new leaves, and these are adapted from the start to a fully autotrophic function (Donnelly and Vidaver 1984).

As ever, economic factors play an important role in *in vitro* propagation. To save time, costs and labor the nurserymen tend to reduce a number of micropropagation stages (Shekhawat and Manokari 2015), by introduction ***ex vitro*** rooting of cuttings. Plants of *Simmondsia chinensis* were produced in 135 days when the cuttings were rooted *ex vitro*; in conventional *in vitro* rooting, 180 days were needed (Singh and Agarwal 2016). According to many authors, the *ex vitro* rooting increases plant survivability during acclimation and further growth in greenhouse as compared with cuttings rooted *in vitro,* and the root system developed *ex vitro* is of better quality (Phulwaria and Shekhawat 2013; Shekhawat and Manokari 2015; Ravindran *et al.* 2016; Singh and Agarwal 2016).

Data on ninebark micropropagation is scarce. Because its cultivars vary in their responses to propagation conditions (Pacholczak and Szydło 2008; Pacholczak 2015), these should often be tailored individually to each one. With this in mind, tests were undertaken to develop an efficient method of micropropagation of for the ninebark cultivar "Dart's Gold," including shortening of the production cycle. Cv. "Dart's Gold" is a popular, hardy, and very decorative shrub not readily rooting when propagated by cuttings. Several factors known to be important in micropropagation were tested, such as different mineral compositions of the media, its concentration, pH, as well as the effects of growth regulators in successive stages of ninebark micropropagation.

Material and methods

1. The experimental material and culture medium The **experimental material** for the experiments were the apical segments of shoots (at least 1.3 cm long) collected randomly from established 8–10 week old *in vitro* cultures of ninebark cv. "Dart's Gold" growing on the MS medium enriched with 1 mg·L^{-1} BA (4.44 μM) and 0.1 mg·L^{-1} NAA (0.54 μM).

The **culture medium** was prepared according to Murashige and Skoog (1962) except in experiment 2 where different media were compared. Sorbitol was used in concentration 30 g· L^{-1}, except in experiment 1, where two carbohydrates were compared. Media were solidified with 8.0 g·L^{-1} Bacto™ Agar (Sparks, Maryland), and their pH at 5.8 before autoclaving was adjusted with 1 N NaOH and 1 N HCl—except in experiment 3 where four pH levels were compared. Media were portioned into 450 ml jars, 50 ml in each and autoclaved for 20 min at 121°C and 110 kPa. There were 10 replicates (jars) each containing 5 explants, i.e., in total 50 explants per treatment. The jars with explants were placed in a phytotron under 23 ± 1°C 16 h day with light of 35 μmol·m^{-2}·s^{-1} photosynthetic photon flux (PPF), provided by fluorescent lamps. The relative humidity was maintained as 55–65%. After 8 weeks of culture, the percentage of regenerating explants, numbers of shoots per explant, and root lengths (cm) were scored.

Multiplication To assess the effect of the **carbon source** on multiplication rates (experiment 1), the explants were placed on the MS medium with either fructose or sorbitol, in concentrations: 1, 2, 3, 4, or 5%. The control treatment was the medium without sugar.

The effect of **macronutrients** on the multiplication rates (experiment 2) was assessed on the following culture media: AN (Anderson 1980), MS (Murashige and Skoog 1962), NN (Nitsch and Nitsch 1969), QL (Quoirin and Lepoivre 1977), SH (Schenk and Hildebrandt 1972), and WPM (Woody Plant Medium - Lloyd and McCown 1980). Vitamins and micronutrients were added according to the MS. All media were supplemented with 1 mg·L^{-1} BA and 0.1 mg·L^{-1} NAA. The effect of **macronutrient concentrations** on the multiplication rates (experiment 3) was tested on four MS variants: ½MS, MS, 1½MS, and 2MS. The effect of **pH** (experiment 4) was tested on MS media at pH 5.4, 5.8, 6.2, and 6.6 as measured before autoclaving.

The effect of a **cytokinin and its concentration** was evaluated in Experiment 5 where the MS medium was supplemented with 0.54 μM NAA and the following cytokinins: 2iP, BA, kinetin, meta-Topolin, TDZ, and zeatin in the following concentrations: 0.5, 1.0, 2.0, or 3.0 mg·L^{-1}. The control treatment was the MS medium without growth regulators. The effect of the **gibberellic acid** on shoot proliferation and elongation (experiment 6) was tested on MS supplemented with 0.1 mg·L^{-1} NAA (0.54 μM), 1 mg·L^{-1} BA (4.44 μM) and GA_3 in concentration: 0, 0.25, 0.5, 1.0 or 2.0 mg·L^{-1}. The MS medium without growth regulators served as control.

The effect of the **culture age,** i.e., passage number, on the multiplication ratio was tested throughout the duration of the entire experiment and included 20 successive passages (Experiment 7). Each passage started with explants placed on the MS medium supplemented with 0.1 mg·L^{-1} NAA and 1 mg·L^{-1} BA. Plants were transferred to fresh medium after every 8 wk of culture. For each successive passage five shoots were taken from each jar (50 in total).

Rooting The material was collected from 8 to 10 week old *in vitro* cultures growing on MS supplemented with 1 mg·L^{-1} BA and 0.1 mg·L^{-1} NAA. Apical parts of regenerated microshoots at least 1.5 cm long were placed onto the rooting medium (MS). After 8 weeks, the following parameters were scored: % of rooted cuttings, mean number of roots per cutting, and mean root length. Each treatment contained 10 replications with 5 plants in each (50 cuttings in total).

To test the effect of **macronutrient concentration** in the rooting medium (Experiment 8), the explants were placed onto the medium containing 1 mg·L^{-1} (4.92 μM) IBA and different concentrations of the MS macronutrients (Table 1). The other media components were as in the original MS formulation.

To test the effects of the **type and concentration of auxin** (Experiment 9), cuttings were placed onto a one-half concentration MS with auxins IBA or NAA in concentrations: 0.1, 0.5, 1.0, 2.0, and 3.0 mg·L^{-1}, while the control MS medium did not contain auxins.

Acclimation The material for test of **acclimation** came from 8 to 10 week cultures on one half MS with 4.92 μM IBA (experiment 10). Plants were at least 3.5 cm tall with 9 internodes and 4–6 roots not shorter than 1 cm each. Plants were removed from the jars, and their roots were rinsed with distilled water to remove the culture medium and potted into M9 pots (9 cm in diameter) with sterile mixture of white peat and perlite (1:2 *v*/v). The pots were placed in transparent containers (Curver 33 l) and treated with fungicides (0.15% Topsin SC + 0.2% Captan). The containers were closed with transparent covers to maintain stable humidity and placed in a greenhouse at 25/20°C (day/night) and RH 50%. After 1 week the covers were gradually removed to allow plants to acclimatize to the *ex vitro* conditions. Eight weeks after potting the percentage of acclimatized plants was scored, their height measured and internode number counted. Each treatment contained 5 replicates with 10 plants each (50 plants in total).

To improve the acclimation, plants were sprayed with **ABA**: 0.5, 1, or 2 mg·L^{-1} (1.89, 3.78 or 7.57 μM) (experiment 11). The effect of the treatment was assessed after 8 wk under greenhouse conditions. The effect of **ABA** was also tested in combination with supplementary lightning (experiment 12). Tested plants were transferred to the greenhouse and sprayed with 1 mg·L^{-1} ABA or water, and for the following 8 weeks, one half of the plants were illuminated between 2 and 9 p.m. with sodium lamps with light intensity of 230 μmol·m^{-2}·s^{-1} (measured with Radiometer-Photometer RF-100 Sanopan). The other half of plants were grown without supplementary lightning.

Table 1 Macronutrient composition of the MS media used in different concentrations

Macronutrients (mg·L^{-1})	Media		
	¼ MS	½ MS	MS
NH_4NO_3	412.5	825.0	1650.0
KNO_3	475.0	950.0	1900.0
$CaCl_2$	83.0	166.0	332.0
$MgSO_4$	45.1	90.3	180.5
KH_2PO_4	42.5	85.0	170.0

***Ex vitro* rooting** A test was undertaken to combine **simultaneous rooting and acclimation** to the *ex vitro* conditions (Experiment 13). The cuttings were taken out of the jars, cut to 2.5 cm in length and soaked for 15 min either in distilled water (control treatment) or in water solutions of 1 or 2 mg·L^{-1} IBA (Table 2), and planted into Styrofoam boxes filled with the mixture of white peat and perlite (*v*/v 2:1). The cuttings were then sprayed with distilled water or the solution of 1 mg·L^{-1} ABA. To protect against pathogens, the cuttings were treated with the fungicide mixture: 0.15% Topsin SC plus 0.2% Captan. The boxes were placed in the greenhouse under controlled conditions: 16 h/24 h light, 24/19°C day/night temperature, and RH 50%. They were covered with opaque perforated plastic foil to prevent dessication. Eight weeks after potting, the percentage of acclimatized plants was scored.

Statistical analysis To compare the means, percentages of rooted cuttings were transformed according to Bliss (Wójcik and Laudański 1989) All of the data were subjected to one- or two-factorial ANOVA followed by Duncan's test at $\alpha = 0.05$. The Statgraphics Centurion XVI program was used.

Results

Multiplication The type of sugar in the medium and its concentration significantly affected the *in vitro* regeneration (Table 3). The percentages of regenerating explants were the highest (98–100%) in the control treatment and on the medium supplemented with 1 and 2% fructose or 1–4% sorbitol. The lowest regeneration (60%) was on the medium with 5% sorbitol. The lowest number of axillary shoots was in the control treatment (1.4 per explant) and the highest on the medium enriched with 3% sorbitol (5.6 shoots per explant). The shortest shoots were produced on the medium with 1, 4, and 5% sorbitol (1.9 cm), while the longest were those on the medium with 4% fructose (3.4 cm) (Table 3).

Sorbitol was chosen for the subsequent experiments not only because of its positive effect on the proliferation rate (Table 3) but also because of no effect on media solidification

Table 2 A list of treatments in the experiment on the *ex vitro* rooting

Treatment	Soaking	Spraying
1	Distilled water	Distilled water
2	1 mg · L^{-1} IBA	
3	2 mg · L^{-1} IBA	
4	Distilled water	1 mg · L^{-1} ABA
5	1 mg · L^{-1} IBA	
6	2 mg · L^{-1} IBA	

Table 3 The effect of carbohydrate type and concentration on shoot proliferation in ninebark 'Dart's Gold'. The basal medium was MS supplemented with 1 mg·L^{-1} BA and 0.1 mg·L^{-1} NAA

Carbohydrates	Conc. (%)	Parameters		
		Regenerating explants (%)**	Shoot number	Shoot length (cm)
Control	0	100 e*	1.4 a	2.6 de
Fructose	1	100 e	2.3 bc	2.5 de
	2	100 e	2.5 bc	2.6 de
	3	92 cd	3.9 d	2.7 e
	4	86 bc	4.3 d	3.4 f
	5	86 bc	2.5 bc	2.3 c
Sorbitol	1	100 e	2.9 c	1.9 a
	2	100 e	4.5 d	2.1 b
	3	100 e	5.6 e	2.5 d
	4	98 de	2.9 c	1.9 a
	5	60 a	2.2 b	1.9 a
Mean	93		3.2	2.4

*Means in a *column* followed by the same *letter* do not differ significantly at $\alpha = 0.05$

**100% was 50 explants

as observed in media with fructose. With 2–5% fructose, shoot vitrification and deformations were found, impeding further shoot growth.

The **type of the culture medium** had little effect on the multiplication rate (Table 4). The lowest rate (90%) was observed on the NN medium; all remaining media produced 100% regeneration rate. The highest shoot number was produced on MS (6.1 per explant), while the lowest on QL (1.1 shoot per explant). The longest shoots were on AN (2.9 cm) while the shortest on WPM (1.4 cm). The MS medium appeared to be the best overall and was used in all subsequent experiments.

Table 4 The effect of culture medium on shoot proliferation in ninebark "Dart's Gold"

Treatment medium	Parameters		
	Regenerating explants (%)**	Shoot number	Shoot length (cm)
AN	100 b*	2.8 b	2.9 d
MS	100 b	6.1 d	2.3 c
NN	90 a	2.8 b	2.2 bc
SH	100 b	3.1 b	2.1 bc
QL	100 b	1.1 a	1.9 b
WPM	100 b	4.2 c	1.4 a
Mean	98	3.3	2.1

*Means in a *column* followed by the same *letter* do not differ significantly at $\alpha = 0.05$

**100% was 50 explants

Table 5 The effect of macronutrient concentration in MS medium on shoot proliferation in ninebark 'Dart's Gold'

MS concentration	Parameters		
	Regenerating explants (%)**	Shoot number	Shoot length (cm)
½MS	90 a	2.5 a	1.6 a
1MS	100 b	5.8 c	2.5 c
1½MS	98 ab	5.7 c	2.7 d
2MS	92 a	4.7 b	2.3 b
Mean	95	4.7	2.3

*Means in a *column* followed by the same *letter* do not differ significantly at $\alpha = 0.05$

**100% was 50 explants

Macronutrient concentrations also affected proliferation (Table 5). On MS with at the standard concentration (1x) 100% explants while at one half or 2 strength, the regeneration rate was reduced to 90 and 92%, respectively. The lowest number of shoots was produced on the medium with macronutrients reduced by half (2.5 per explant), and they were the shortest (1.6 cm). The explants cultured on 1 MS and 1½ MS developed the highest number of shoots (over 5.5 per explant), while the longest shoots (2.7 cm) were on 1½ MS.

There was a statistically significant effect of the **medium pH** on the regeneration rate (Table 6). At pH 5.8 and 6.2, all explants regenerated shoots; at pH 6.6 this rate was reduced. The numbers of shoots were the highest at pH 5.4 and 5.8 (5.1 and 5.6, respectively), and the longest shoots (2.3 cm) were produced at pH 5.8 cm.

Table 6 The effect of the medium pH on shoot proliferation in ninebark 'Dart's Gold'. The basal MS medium was supplemented with 1 mg·L^{-1} BA and 0.1 mg·L^{-1} NAA

pH	Parameters		
	Regenerating explants (%)**	Shoot number	Shoot length (cm)
5.4	94 ab*	5.1 b	2.1 b
5.8	100 b	5.6 b	2.3 c
6.2	100 b	3.8 a	1.9 ab
6.6	90 a	3.7 a	1.8 a
Mean	96	4.6	2.0

*Means in a *column* followed by the same *letter* do not differ significantly at $\alpha = 0.05$

**100% was 50 explants

The type and concentration of cytokinin in the culture medium significantly affected the regeneration rate (Table 7, Fig. 1). The lowest percentage of regenerating explants was observed on the medium with 0.1 mg·L^{-1} NAA and supplemented with either 3 mg·L^{-1} kinetin or 3 mg·L^{-1} BA (60% and 72% respectively). In treatments with mT, TDZ, zeatin and 2iP the regeneration rate ranged between 84% and 100% with no significant differences between them. The lowest shoot number per explant, i.e., 1.1–1.2, was in the treatment with auxin only, and on media with kinetin in concentrations: 0.5, 1.0 and 3.0 mg·L^{-1}. The highest proliferation was on the medium with TDZ with number of shoots per explant reaching 19.1.

While TDZ produced the highest numbers of shoots these shoots were very short and malformed, that would impede their separation and transfer to new culture vessels. From the practical point of view, the TDZ effect was regarded as useless and compound was excluded from subsequent experiments. High numbers of shoots were also obtained in treatments with 1 mg·L^{-1} BA or 2 mg·L^{-1} mT in the medium and they were almost 5.5 times higher than the control without growth regulators and with no negative side effects.

Efficient proliferation was also observed on media supplemented with 2 and 3 mg·L^{-1} zeatin (almost 5 times more than in the control). The longest shoots were produced with 2 and 3 mg·L^{-1} zeatin (4.3 and 4.2 cm respectively). Plants taller by nearly 50% than in the control were also produced on the medium with kinetin at all concentrations tested, 0.5–1 mg·L^{-1} meta-Topolin, 1 mg·L^{-1} zeatin, and 2 mg·L^{-1} 2iP (3.0–3.4 cm). Generally, kinetin supplementation increased the shoot length but not shoot formation. The best treatment for both the shoot number and length was the medium with 2 and 3 mg·L^{-1} zeatin.

GA_3 had no effect on the regeneration rate which ranged between 92 and 100% with no significant differences between the treatments (Table 8). However. there was a positive effect of GA_3 on proliferation: the highest shoot number per explant (12.1) was produced at 1 mg·L^{-1} GA_3—almost ten times higher than on media without growth regulators. The shoot lengths (2.1–2.4 cm) were comparable in all treatments.

There was no effect of the **culture age** (the **number of passages)** on the regeneration rate (Table 9). However, the shoot number per explant and shoot length were affected. On the average, explants produced 3.2–6.8 shoots, depending on the passage. The lowest shoot number, which were also the shortest, was in the first passage when the culture has not yet fully established. In the second and third passages, the proliferation rate increased, and then it kept decreasing until the 8th passage. Statistically, the highest numbers of shoots were produced between passages 10 and 17. Between passages 18 and 20, drop in the proliferation rate was again observed. Generally, during the entire experiment, the explants averaged 5.6 shoots. The mean shoot length ranged between 1.7 and 2.3 cm. The shortest shoots were in the first passage, and then their length kept increased until passage 8, reaching 2.3 cm. Similar shoot lengths were produced in passages 12–13 and 18–20.

Table 7 The effect of the cytokinin type and concentration (mg·L^{-1}) on shoot proliferation in ninebark 'Dart's Gold'. The basal medium was MS supplemented with 0.1 mg·L^{-1} NAA; the control was without growth regulators

Treatment		Parameters		
		Regenerating explants (%)**	Shoot number	Shoot length (cm)
Control MS 0		92 cd*	1.1 a	2.1 bc
0.1 NAA		72 ab	1.1 a	2.7 defg
BA	0.5	84 bc	3.1 f	2.1 bc
	1.0	100 d	5.7 j	2.3 cd
	2.0	96 cd	4.3 h	2.3 cd
	3.0	72 ab	3.5 g	1.8 b
Kin	0.5	86 cd	1.1 a	3.1 fgh
	1.0	88 cd	1.2 a	3.1 fgh
	2.0	92 cd	1.6 b	3.2 gh
	3.0	60 a	1.1 a	3.4 h
mT	0.5	88 cd	2.4 de	3.1 fgh
	1.0	84 bc	2.4 de	3.0 fgh
	2.0	96 cd	5.9 j	2.4 cde
	3.0	96 cd	2.6 e	2.0 bc
TDZ	0.5	100 d	17.9 l	0.9 a
	1.0	100 d	18.4 m	1.0 a
	2.0	100 d	19.1 n	0.9 a
	3.0	100 d	16.2 k	0.8 a
Zea	0.5	100 d	2.4 de	1.9 bc
	1.0	96 cd	3.2 f	3.4 h
	2.0	100 d	5.2 i	4.3 i
	3.0	100 d	5.0 i	4.2 i
2iP	0.5	88 cd	2.4 de	2.7 def
	1.0	96 cd	2.3 cd	2.7 def
	2.0	92 cd	2.0 c	3.1 fgh
	3.0	92 cd	2.0 c	2.8 efg
Mean		91	5.1	2.5

*Means in a *column* followed by the same *letter* do not differ significantly at $\alpha = 0.05$

**100% was 50 explants

Rooting Auxin affected all tested parameters of rooting (Table 10, Fig. 2). The lowest percentage of rooted cuttings was observed on the control medium (46%) and on media with 0.1 and 3 mg·L^{-1} IBA (56% and 64%) or 0.1 and 3 mg·L^{-1} NAA (62% in both treatments). The highest rooting percentage was on media supplemented with either 1 and

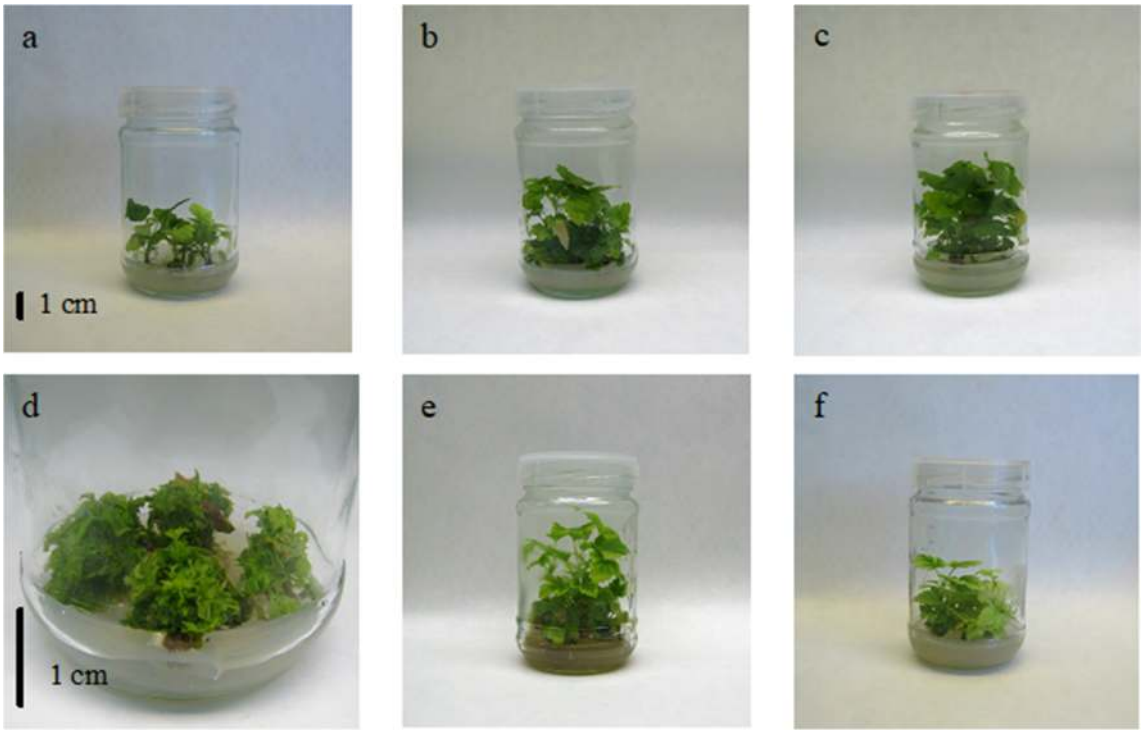

Figure. 1 Proliferating shoots of ninebark "Dart's Gold" after 8-week culture on the medium: (**a**) without growth regulators (control); supplemented with: (**b**) 1 mg·L^{-1} BA; (**c**) 2 mg·L^{-1} mT; (**d**) 1 mg·L^{-1} TDZ; (**e**) 2 mg·L^{-1} Zea; (**f**) 1 mg·L^{-1} 2iP

2 mg·L^{-1} IBA (96% and 92%) or 1 and 2 mg·L^{-1} NAA (88% and 96%). The highest root numbers per cutting were produced in the presence of 3 mg·L^{-1} IBA or NAA (13 and 12 roots), the lowest on the control medium (3.3 root), or in the presence of 0.1 mg·L^{-1} IBA (4.3 roots). The longest roots were produced on the medium with 0.1 mg·L^{-1} IBA (1.3 cm), while the shortest in the presence of 0.1, 0.5, 1, and 3 NAA mg·L^{-1} (0.2–0.3 cm).

The **macronutrient concentration** significantly affected rooting parameters (Table 11). On the medium with one fourth MS only, 60% cuttings developed roots and those roots were fewer (3.5 roots per cutting) and shorter (0.4 cm) than in cuttings cultured on other media. Plants from one half MS and MS did not differ in the percentage of rooted cuttings (96%) nor in mean root number and length (8.8 and 8.9 roots 1.2 and 1.3-cm-long, respectively).

Spraying plants with ABA significantly affected all **acclimation** parameters tested (Table 12). In control treatments, only 80% of plants became adapted to the greenhouse conditions, while in all other treatments, all plants were successfully adapted. The shortest were plants sprayed with 0.5 and 2 mg·L^{-1} ABA, and their height was approximately 93% of that in control plants. The highest were plants sprayed with 1 mg·L^{-1} ABA (9 cm on the average). The lowest internode

Table 8 The effect of GA_3 on shoot proliferation in ninebark "Dart's Gold"

No	NAA (mg ·L^{-1})	BA (mg ·L^{-1})	GA_3 (mg·L^{-1})	**Parameters**		
				Regenerating explants (%) **	Shoot number	Shoot length (cm)
1	0	0	0	92 a*	1.3 a	2.3 a
2	0.1	1.0	0	100 a	6.1 b	2.4 a
3			0.25	96 a	8.5 c	2.2 a
4			0.5	100 a	11.0 d	2.3 a
5			1.0	100 a	12.1 e	2.1 a
6			2.0	96 a	9.0 c	2.1 a
Mean				97	8.0	2.2

*Means in a *column* followed by the same *letter* do not differ significantly at $\alpha = 0.05$

**100% was 50 explants

Table 9 The effect of the number of passages on shoot proliferation in ninebark "Dart's Gold." The basal medium was MS supplemented with 1 mg·L^{-1} BA and 0.1 mg·L^{-1} NAA

Passage number	Parameters		
	Regenerating explants (%)**	Shoot number	Shoot length (cm)
1	98 a*	3.2 a	1.7 a
2	100 a	5.5 def	2.3 de
3	100 a	5.6 efg	2.3 de
4	100 a	4.8 bc	2.2 cde
5	100 a	4.5 b	2.3 e
6	100 a	4.3 b	2.2 cde
7	100 a	4.7 bc	2.2 cde
8	100 a	4.9 bcd	2.3 e
9	100 a	5.4 cde	2.1 bcd
10	100 a	6.8 j	1.9 b
11	100 a	6.8 ij	1.9 b
12	100 a	6.6 ij	2.2 cde
13	98 a	6.8 j	2.3 e
14	100 a	6.2 hij	1.9 b
15	100 a	6.3 ij	2.0 bc
16	100 a	6.6 ij	2.0 bc
17	100 a	6.1 ghi	2.0 bc
18	100 a	5.4 cde	2.3 e
19	100 a	5.2 cde	2.1 bcde
20	98 a	5.3 cde	2.2 cde
Mean	99.7	5.6	2.1

*Means in a *column* followed by the same *letter* do not differ significantly at $\alpha = 0.05$

**100% was 50 explants

Table 10 The effect of auxin on rooting of axillary shoots in ninebark "Dart's Gold"

Treatment (mg·L^{-1})	Parameters		
	Rooted cuttings (%)**	Root number	Root length (cm)
Control	46 a	3.3 a	1.2 d
0.1 IBA	56 a	4.3 a	0.6 bc
0.5 IBA	82 cd	6.2 b	0.8 c
1 IBA	96 e	9.2 c	1.3 e
2 IBA	92 de	10.0 c	0.7 bc
3 IBA	64 ab	13.0 d	0.6 b
0.1NAA	62 ab	6.2 b	0.3 a
0.5 NAA	76 bc	5.9 b	0.3 a
1 NAA	88 de	6.0 b	0.2 a
2 NAA	96 e	9.0 c	0.5 b
3 NAA	62 ab	12.0 d	0.3 a
Mean	75	7.7	0.6

*Means in a *column* followed by the same *letter* do not differ significantly at $\alpha = 0.05$

**100% was 50 explants

number was found in plants treated with 2 mg·L^{-1}, ABA while this value did not differ in other treatments.

The effect of spraying with ABA and of supplementary lightning on acclimation to the *ex vitro* conditions is shown in Table 13. The lowest percentage of acclimatized plants (76%) was in plants not given extra light and sprayed with water. In all remaining treatments, all plants were properly acclimated. Two months after planting the smallest plants with the lowest internode number were observed in the treatment where plants were sprayed with ABA and not given extra light: the average height of plants was 7.4 cm and they had 9.4 internodes. The best growth was in plants sprayed with ABA and grown under supplementary lighting: they were 14.3-cm tall and averaged 12.2 internodes.

Treatments with IBA and ABA significantly affected **rhizogenesis** *ex vitro* (Table 14, Fig. 3). The lowest percentages of rooted and acclimated plants were in treatments where cuttings were soaked in water and sprayed with water or ABA (30 and 38%, respectively). The highest percentage of cuttings rooted *ex vitro* was in plants soaked in the solution of 1 mg·L^{-1} IBA and sprayed with distilled water (80%) as well as in plants soaked in 1 and 2 mg·L^{-1} IBA and sprayed with 1 mg·L^{-1} ABA (74 and 64%). Significantly fewer roots were produced by cuttings soaked in water and sprayed with water or ABA, as well as those soaked in 2 mg·L^{-1} IBA and then sprayed with ABA. The highest root numbers were observed after soaking in 1 or 2 mg·L^{-1} IBA and spraying with water (3.6 roots per plant). The shortest roots were produced in plants soaked in water and sprayed with water and in those soaked in 2 mg·L^{-1} IBA and sprayed with ABA (0.8 and 0.9 cm, respectively). The longest roots developed in cuttings soaked in 1 mg·L^{-1} IBA and sprayed with water (1.7 cm) and in those soaked in water and sprayed with ABA (1.5 cm).

Discussion

The main goal of micropropagation is to obtain quickly and as efficiently as possible, a maximal amount of good quality plant material. Numerous factors affect the efficiency of the process, such as the type and concentration of carbohydrates, macronutrient composition, medium pH, composition and dosages of growth regulators as well as duration and the number of passages, individually and in combinations. Overall, the optimization of the process can be rather tedious.

According to Ilczuk et al. (2013), the most suitable **carbohydrate source** for micropropagation of ninebark cv. "DIABLE D"OR "Mindia" was fructose; its presence in the medium yielded the highest percentage of regenerating

Figure. 2 Plants of ninebark 'Dart's Gold' after 8 wk of rooting on the medium: (**a**) control, without auxin; (**b**) supplemented with 1 mg·L^{-1} IBA; and (**c**) with 1 mg·L^{-1} NAA

explants relative to glucose, maltose, and sucrose. Fructose also performed the best during the rooting stage. However, media with fructose often lead to vitrification and this reduces plant vigor. This was reported earlier by Pierik (1997). Moreover, problems with solidifying media containing fructose were observed during ninebark multiplication (Ilczuk *et al.* 2013), perhaps due to fructose degradation during autoclaving and appearance of toxic furans (Rédei 1979). Here, another carbohydrate source, sorbitol, was tested, and it resulted in good proliferation while avoiding the solidification issues. According to Ahmad *et al.* (2007) and Yaseen *et al.* (2009), many plants from the Rosaceae family multiply well in the presence of sorbitol. For cv. "Dart's Gold" tested here, the best medium was the MS with 3% sorbitol, producing 100% regeneration and the highest shoot number per explant. Similar results were obtained by Ahmad et al. (2007) on peach and by Yaseen et al. (2009) on apple rootstocks M9 and M26. The positive effect of sorbitol in the *Rosaceae* may be related to the fact that it is their main photo assimilate (Moing *et al.* 1992) or because the enzymes responsible for its hydrolysis are already in place (Ahmad *et al.* 2007). The activity of such enzymes was confirmed in tissues of the sorbitol-translocating plants (Stoop and Pharr 1993).

The nutrient composition of a culture medium is decisive for the number and quality of shoots produced in the multiplication phase. Cv. "Dart's Gold" tested here showed the best proliferation on the MS even though WPM is often reported as the most suitable medium for woody plants. Similar results were obtained by Kassim et al. (2010) on *Prunus amygdalus* or by Gallo *et al.* (2017) on the rootstock *Prunus* "Mr.S.2/5," where MS gave significantly better proliferation than WPM or other media. This may be a consequence of a higher macronutrient contents of MS. NH_4^+ ions included. According to Mercier *et al.* (1997) the nitrogen source (NO_3^-, NH_4^+) directly affects the levels of endogenous IAA and cytokinins; hence, differences in salt concentrations may induce different plant responses during the multiplication phase (Matt and Jehle 2005).

The **salt concentration** in the medium is decisive for shoot proliferation. Here, the proliferation rate was the highest on MS at full concentration, as well as on 1½ MS, the latter resulting also in the longest shoots. On the other hand, reduced salt concentration such as in ½ MS or increased concentration to 2 s reduced the regeneration rate by approx. 10%. The lowest shoot number was produced on the medium with the macronutrients reduced by one half and they were the shortest. However, in some leguminose trees, the half-strength MS medium worked well (Yuji *et al.* 1993); in dogwood, there was no difference in shoot numbers between 1x and ½ MS (Declerck and Korban 1996).

The medium pH affects the availability and uptake of nutrients by plant tissues, and sometimes a small change in pH may significantly affect plant growth, instead towards

Table 11 The effect of macronutrient concentrations on rooting of shoots in ninebark "Dart's Gold"

MS concentration	Rooted cuttings (%)**	Parameters Root number	Root length (cm)
1/4 MS	60 a	3.5 a	0.4 a
1/2 MS	96 b	8.8 b	1.2 b
MS	96 b	8.9 b	1.3 b
Mean	84	7.1	1.0

*Means in a *column* followed by the same *letter* do not differ significantly at $\alpha = 0.05$

**100% was 50 cuttings

Table 12 The effect of spraying *in vitro* rooted ninebark cuttings with ABA on their acclimation to the *ex vitro* conditions

Treatment – ABA (mg·L^{-1})	Acclimatized plants (%)**	Parameters Plant height (cm)	Internode number
Control - 0	80 a*	8.4 b	9.4 b
0.5	100 b	7.7 a	9.2 b
1.0	100 b	9.0 c	9.4 b
2.0	100 b	7.9 a	8.8 a
Mean	95	8.3	9.2

*Means in a *column* followed by the same *letter* do not differ significantly at $\alpha = 0.05$

**100% was 50 cuttings

Table 13 The effect of ABA (1 mg·L^{-1}) and supplementary lighting on ninebark acclimation to the *ex vitro* conditions

Treatment		Parameters		
Sprayed with	Lighted	Acclimatized plants (%)**	Plant height (cm)	Internode number
H_2O	–	76 a*	8.0 b	10.1 b
ABA		100 b	7.4 a	9.4 a
H_2O	+	100 b	13.3 c	11.6 c
ABA		100 b	14.3 d	12.2 d
Mean		94	10.8	10.8

*Means followed by the same *letter* do not differ significantly at $\alpha = 0.05$

**100% was 50 cuttings

growth. The energy is directed towards adaptation to the medium pH (Thorpe *et al.* 2008). According to Javed et al. (2013), the medium pH significantly affected multiplication in *Acacia ehrenbergiana* with optimum at pH 5.8. The same pH worked well in magnolia (Cui *et al.* 2019). In *Amygdalus communis* pH 5.5 was the best for the initiation and multiplications phases; lower and higher pH produced some disorders (Gürel and Gülşen 1998). Here, in "Dart's Gold," the multiplication ratio and shoot length were higher at lower pH (5.4–5.8).

Cytokinin type and concentration do have specific effects on organogenesis *in vitro* in various taxa; their uptake, transport, and metabolism differ among species, cultivars, or even tissues. As cytokinins induce cell divisions, hence bud and shoot formation, they are indispensable in micropropagation (Davies 2010). Among various cytokinins, benzyladenine (BA) is one of the most efficient and is readily available (Werbrouck *et al.* 1996). In this study, both the type and concentration of cytokinin affected shoot proliferation.

Table 14 The effect of treatments with IBA (mg·L^{-1}) and ABA (mg·L^{-1}) on the *ex vitro* rooting and acclimation to greenhouse conditions of cuttings in ninebark "Dart's Gold"

Treatment		Parameters		
Soaking	Spraying	Rooted cuttings (%)**	Root number	Root length (cm)
H_2O	H_2O	30 a	2.5 a	0.8 a
1 IBA		80 c	4.2 c	1.7 c
2 IBA		50 ab	4.2 c	1.2 b
H_2O	1 ABA	38 a	2.5 a	1.5 c
1 IBA		74 bc	3.6 bc	1.2 b
2 IBA		64 bc	2.9 ab	0.9 ab
Mean		56	3.3	1.2

*Means in a *column* followed by the same *letter* do not differ significantly at $\alpha = 0.05$

**100% was 50 cuttings

Figure. 3 Cuttings of ninebark 'Dart's Gold' after 2 weeks of *ex vitro* rooting

The highest multiplication ratio was observed with TDZ, but resulting plants were shorter and differed morphologically from standard phenotype, and this cytokinin was dropped from further tests. Similar abnormalities were observed in *Cornus florida* (Kaveriappa *et al.* 1997) or *Cotoneaster wilsonii* (Sivanesan *et al.* 2011). In this study high numbers of good quality shoots were obtained on medium with 1 mg·L^{-1} BA and 2 mg·L^{-1} mT while the longest shoots were produced in the presence of 2–3 mg·L^{-1} zeatin. A positive effect of mT on regeneration was reported in pear rootstock OHF-333 (Dimitrova *et al.* 2016) and smoke tree "Royal Purple" (Podwyszyńska *et al.* 2012). Explants of *Pelargonium* placed on the mT-containing medium produced twice as many shoots as those on BA (Wojtania 2010). Zeatin affects not only shoot elongation as here in ninebark, in olive cultivars (Micheli *et al.* 2010), and apricot (Murai *et al.* 1997) but also increases the multiplication ratio as in *Cissus quadrangularis* (Garg and Malik 2012). For efficient propagation of a given taxon, different cytokinin concentrations may be suitable. In *Catalpa ovata*, a high zeatin concentration of 5 mg·L^{-1} was needed to stimulate proliferation (Lisowska and Wysokinska 2000). In *Acacia ehrenbergiana*, three cytokinins tested (BA, Kin, and 2iP) were the most efficient when used in concentration 2 mg·L^{-1}. The authors suggested that optimal concentrations of different cytokinins are comparable as there is a threshold cytokinin concentration which can be tolerated by plant. When exceeded, it provokes a plant defense response which needs energy normally used for shoot growth and development (Javed *et al.* 2013). Our results are not in line with this speculation as an optimal concentration depended on a cytokinin type. The best proliferation in ninebark "Dart's Gold" occurred by the following concentrations: 1 mg·L^{-1} BA, 2–3 mg·L^{-1} Kin, 2 mg·L^{-1} mT, 2–3 mg·L^{-1} TDZ, 2 mg·L^{-1} Zea, and 0.5–1 mg·L^{-1} 2iP. Because of the low concentration needed and lack of subsequent problems with rooting, benzyladenine was chosen for the further experiments in

ninebark as the most cost-effective of all the cytokinins tested. Earlier it proved suitable for the cultivar "DIABLE" OR "Mindia" (Ilczuk *et al.* 2013).

Low concentration of NAA stimulated the cytokinin-induced proliferation in ninebark. Similarly, a synergetic influence of BA and NAA was noticeable in *Ruta graveolens* when the combinations of the most favorable concentration of BA with variant concentrations of NAA were tested: supplementation of NAA resulted in a better shoot proliferation rate and the best regeneration (Faisal *et al.* 2018). In *Juglans regia* the best proliferation results were obtained with 1 mg·L^{-1} of BA plus 0.01 mg·L^{-1} of IBA (Kepenek and Kolagasi 2016). During multiplication phase the auxin IAA or IBA was always present in culture media used for seven ornamental plants differing in sensitivity towards cytokinins BA or Kin (Vardja and Vardja 2001).

In most cases, shoot elongation is enhanced by GA (Gupta and Chakrabarty 2013), but not in the ninebark tested here. GA did not affect shoot growth but shoot proliferation, and the highest shoot number per explant were obtained with 1 mg·L^{-1} GA_3. Similar effect was reported for five raspberry cultivars (Zawadzka and Orlikowska 2006), apricot (Koubouris and Vasilakakis 2006), and lavender (Machado *et al.* 2011).

With time spent *in vitro*, senescence symptoms become frequent, mutations appear, and epigenetic changes may accumulate, reducing the multiplication ratio, lowering plant vigor, or inducing changes in plant morphology (Huang *et al.* 2012). Ninebark cultures and their regeneration ability were observed during 20 subcultures, and no substantial differences in the percentage of explants regeneration on the same medium were noted. The lowest numbers of shoots *per* explants and the shortest shoots were in the first subculture. Similar observation was made by Javed et al. (2013) on *Acacia ehrenbergiana*, and it was explained by the effect of the oxidative stress after mechanical wounding of plant material during culture establishment. Apart from this, at the beginning of culture, the explants use part of their energy to adapt to new conditions *in vitro* instead of spending it on growth. Similarly, in *Rubus pubescens*, the regeneration parameters were the lowest in the first subculture; in the second and third passage, they improved and remained on the same level during successive subcultures (Debnath 2004). Another tendency was reported by Remphrey et al. (1993) who studied the effect of the long-term culture on regeneration in *Potentilla fruticosa*: regeneration potential peaked in the second passage and declined during successive subcultures until the tenth passage. Also, in rootstocks of fruit trees, the regeneration ability declined during the long-term culture including 10 subcultures (Vujović *et al.* 2012). Sometimes it is recommended to reduce by half the BA content of the multiplication medium after the 5th or 6th subculture or to transfer a culture onto media of lesser salt content from time to time (Vardja and Vardja 2001). In ninebark "Dart's Gold," the highest shoot numbers *per* explant were produced on the same medium between passages 10 and 17, and there was no relationship between the culture age and the shoot length even after 20 successive subcultures, i.e., for over 3 years.

In the **rooting phase** of micropropagation, the culture medium is designed to produce well-developed root systems in cuttings. Not only the mineral composition of the medium is decisive, but also, the nutrient concentration is important. Excessive amounts of salts may limit root development; hence, media with lowered salt concentrations are often used. According to Hong (1996), explants of *Rosa chinensis* rooted better on one half MS than on 1x MS. Similar observations were made by Kikowska et al. (2014) on *Eryngium maritimum*, by Fadel et al. (2010) on *Mentha spicata*, and by Bidarigh and Azarpour (2013) on *Camellia sinensis.* In this study, there was no difference between one half and 1x MS so a reduction of the MS concentration reduces the cost of the process without affecting the outcome.

Auxin is the main hormone controlling **rhizogenesis**. Exogenous auxin in a culture medium may affect enzyme activities in tissues (IAA oxidase and peroxidase) and phenolic compounds contents, thus creating a hormone balance suitable for root initiation (Qaddoury and Amssa 2004). Auxin activates root primordia or stimulates their mitotic activity (De Klerk *et al.* 1999). Among various auxins used to stimulate rooting IBA is the most commonly used, IAA and NAA are considered as less suitable (Hartmann *et al.* 2011) but much appears to depend on the species involved. Ahn *et al.* (2007) obtained comparable results with IBA and NAA in *Ricinus communis*; however, plants rooted in the presence of IBA showed a higher rate of establishment in the soil (93.5 *vs* 39.5%) and a better growth. In *Prunus armeniaca* (Koubouris and Vasilakakis 2006) and *P. dulcics* (Tereso *et al.* 2008) rooting was better with IBA, IAA, or IBA than NAA simillary as in *Acacia chundra* (Rout *et al.* 2008). In an apple rootstock "Jork 9" IAA and IBA gave comparable results when used at optimal concentrations. However, IAA was effective in a concentration range between 1.8 mg·L^{-1} and 17.5 mg·L^{-1} (10–100 μM IAA), while IBA had only one optimal value, i.e., mg·L^{-1} (10 μM) (De Klerk *et al.* 1999). In this study, IBA produced better rooting than NAA, but as in some other studies (De Klerk *et al.* 1999), the range of effective concentrations was narrow.

Acclimation is the last phase of micropropagation when plants produced *in vitro* are gradually adjusted to external conditions. Various conditions *in vitro* may result in development of plants with altered morphology, anatomy, and physiology. A transfer to external environment may result in damage and physiological disorders. Usually, stomata of *in vitro*-generated plants do not function normally, so a reduction of the transpiration rate is crucial for plant hardening. Antitranspirants such as ABA are generally used to hasten acclimation as they close stomata and reduce water losses

(Pospíšilová *et al.* 1999; Rai *et al.* 2011). ABA can be delivered in a different ways, *via* spray or a media supplement. As a media supplement, it increased the survival rate in *Tagetes erecta* and increased leaf area (Aguilar *et al.* 2000). Mulberry plants treated with ABA during the last subculture acclimatized in a higher percentage and had better growth parameters than untreated plants (Huh *et al.* 2017). In this study, cuttings sprayed with ABA acclimatized in a higher percentage than those sprayed with water. After 2 months of acclimation, they were also taller. Another important factor in acclimation is light. Leaves developed *in vitro* are usually unable to produce sufficient amounts of metabolites necessary for normal development. Increased light intensity during acclimation hastens growth of new leaves which are fully autotrophic (Donnelly and Vidaver 1984). Here, supplementary lighting during acclimation significantly increased plant height and internode number.

Recently, a particular emphasis is placed on *ex vitro rooting* of microcuttings as it saves time, costs, and labor of plant production (Shekhawat and Manokari 2015). Not only the production cycle can be shortened, but more plants survive the transfer from *in vitro* to *ex vitro* conditions, and the acclimation to greenhouse conditions proceeds simultaneously with the *ex vitro* rooting (Phulwaria and Shekhawat 2013; Shekhawat and Manokari 2015; Ravindran *et al.* 2016; Singh and Agarwal 2016). The percentage of plants of *Vaccinium corymbosum* L. "Berkeley" and *V. angustifolium* × *V. corymbosum* "Northsky" rooted and acclimatized *ex vitro* was nearly 100% (Isutsa *et al.* 1994). In the difficult-to root magnolia, the percentage of rooted cuttings and the mean root number were higher under the *ex vitro* conditions relative to *in vitro* (Parris *et al.* 2012). *Ex vitro* rooting of ninebark microcuttings was successful in our trials with 80% success rate after soaking them in a water IBA solution. As ABA increases plant tolerance to adverse conditions, it is usually used to improve acclimation of the *ex vitro*-rooted microcuttings. Twice as many cuttings of *Jatropha curcas* rooted *ex vitro* after a pretreatment with ABA as compared with untreated cuttings (Singh *et al.* 2014). However, in this study no effect of sprayed ABA was observed.

Conclusions

Overall, this study offers an effective and efficient protocol for micropropagation of ninebark. Reduction in media concentrations, adjustments in growth regulator combinations, and direct rooting of cuttings during acclimation process reduce the overall cost of the process and make it much more efficient. Unfortunately, it appears quite likely that individual steps and the component compositions may have to be tailored to individual cultivars. Only such protocols may help to satisfy the growing demand for this ornamental shrub. The following conclusions might be helpful in micropropagation of ninebark "Dart's Gold.'

1. 1x MS basal medium, pH 5.8, supplemented with 2% sorbitol produced the best regeneration rates in ninebark "Dart's Gold."
2. The type and concentration of cytokinin in the culture medium significantly affected regeneration. Benzyladenine at 1 mg· L^{-1} proved the most cost-effective cytokinin, while the presence of gibberellic acid in the culture medium containing NAA and BA further increased proliferation.
3. There was no effect of the culture age (up to 20 subcultures) on the percentage of regenerating explants.
4. One half MS macronutrient concentration was sufficient for rhizogenesis which was better stimulated by the auxin IBA than NAA.
5. Spraying the *in vitro* rooted cuttings with ABA favored plant acclimation to the *ex vitro* conditions.
6. The *ex vitro* rooting was successful in reducing the production time by a third.

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