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ORIGINAL PAPER

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ROOTING SHOOTS OF APPLE VARIETIES AND THEIR TETRAPLOIDS OBTAINED BY THE *in vitro* TECHNIQUE

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ABSTRACT

For breeding purposes, the number of neo-tetraploids of apple cultivars have been derived by *in vitro* technique. The first attempts at rooting and acclimatization of tetraploid shoots failed. The aim of the study was to develop an effective method for rooting microcuttings of apple neo-tetraploids. In the first stage of the study, *in vitro* rooting method was optimized for the shoots of diploid donor cultivars. Shoots were rooted on Murashige and Skoog [1962] (MS) medium with a reduced content of nitrogen, in the presence of auxins alone or in combination (indole-3-butyric acid – IBA, 1-naphthaleneacetic acid – NAA and indole--3-acetic acid – IAA) with addition of putrescine, arginine or ornithine. The compounds were applied continuously for 25 days (one-step rooting system) or for seven days with subsequent transplanting shoots onto a medium without these compounds (two-step rooting method). Tetraploid microcuttings of the cultivars 'Free Redstar', 'Gala Must', 'Pinova' and 'Redchief' were evaluated for rooting on the selected medium considered optimal for their diploid counterparts. The shoots of all diploid apple scion cultivars had low rooting capacity. IBA alone poorly stimulated root formation. Significant improvement of rooting to 60–80% was achieved through the application of auxins, 2.5 µM IBA or 1.3 µM NAA combined with 5 µM IAA and 50 µM putrescine in the two-step rooting system with darkness and increased temperature of 26°C during seven-day induction phase. The replacement of benzyladenine (BA) by *meta*-Topolin (*m*-T) in the last multiplication subculture influenced positively shoot acclimatization. Tetraploids had comparable or slightly lower rooting and acclimatization ability compared to their diploid counterparts.

Key words: *Malus* × *domestica*, tetraploids, auxins, *meta*-Topolin, putrescine, arginine, darkness

INTRODUCTION

Among several thousand apple varieties, there are approximately 10% of triploids and just a few tetraploids [Janick et al. 1996, Podwyszyńska et al. 2016]. Triploid or tetraploid varieties are characterized by a number of valuable features: larger fruits and often greater resistance to biotic and abiotic stress [Sedysheva and Gorbacheva 2013]. In the Research Institute of Horticulture (Skierniewice, Poland) for breeding purposes, tetraploids of five cultivated apple cultivars were obtained by *in vitro* mitotic polyploidization [Podwyszyńska et al. 2017]. Shoots of the newly obtained tetraploids differed significantly from those of their diploid counterparts. Tetraploid shoots were shorter, more robust and had dark green leaves (fig. 1A). The first attempts at rooting and acclimatization of tetraploid shoots failed. Moreover, the results of preliminary experiments showed that microcuttings of all tested donor apple cultivars had very low rooting ability. It was well documented that shoots of micropropagated apple

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scion cultivars produced roots poorly compared to rootstock genotypes [Lane and McDougald 1982]. Dobránszki and Texeira da Silva [2010] concluded that differences in rooting capacity between rootstocks and scions were the consequence of selective pressure during breeding process since high rooting frequency of cuttings is the desirable feature of rootstocks. Several shoot rooting procedures, including *in vitro* and *ex vitro* methods were reported for apple cultivars propagated *in vitro* [Dobránszki and Texeira da Silva 2010]. Rooting of micropropagated shoots is an essential process in *in vitro* propagation [Moncousin 1991]. Adventitious root formation *in vitro* has been studied extensively in many species, e.g. in mung bean [Nag et al. 2001] and apple [De Klerk et al. 1999]. Generally, adventitious root formation both *in vitro* and *in vivo* can be divided into four phases and in apple the process is as follows: 1) induction (dedifferentiation phase, lasting approximately 24 h, some cells close to the vascular tissue turn meristematic and sensitive to auxin-inducing rhizogenesis; 2) initiation (next 2-4 days) when competent cells

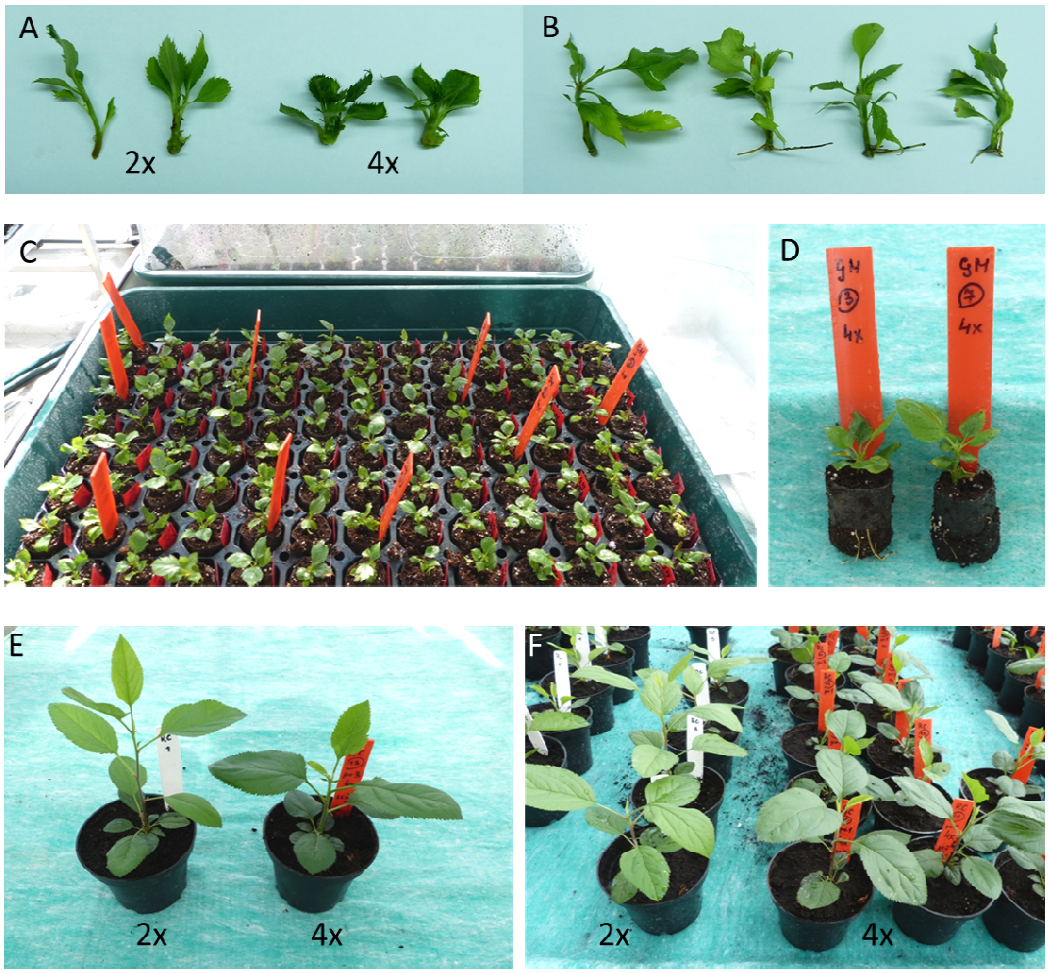

Fig. 1. Shoot rooting *in vitro* and acclimatization to *ex vitro* conditions of diploid apple cultivars and their tetraploid counterparts: diploid and tetraploid shoots of 'Free Redstar' (A); rooted shoots of donor cultivar 'Gala Must' using two step rooting system, treatments from the left (µM): control, IBA 2.5 + IAA 5, IBA 2.5 + IAA 5 + arg 250, IBA 2.5 + IAA 5 + put 50 (B); diploid and tetraploid plants of 'Gala Must' four weeks (C and D) and 8 weeks (E and F) after transplanting to *ex vitro* conditions

respond to auxins and begin to divide forming meristematic centers named meristemoids; 3) formation of root primordia from meristemoids at 4th or 5th day after placing shoots on rooting medium, at this time auxins are no longer required, 4) root elongation, appearing after 5–6 days as root tips emergence through tissue at the base of the shoot [De Klerk et al. 1999]. Auxins play a key role and their presence in a medium is required only for a few days for rhizogenesis induction [Kevers et al. 1997]. As reviewed by Dobránszki and Texeira da Silva [2010], the most commonly auxins used for rooting shoots of apple were indole-3-butyric acid (IBA) and indole--3-acetic acid (IAA); 1-naphthaleneacetic acid (NAA) was utilized less frequently. The auxins were applied at various concentrations IBA at 0.1–5 mg l^{-1}, IAA at 0.3–20 mg l^{-1} and NAA at 0.3–2 mg l^{-1}. Root formation of apple microcuttings is influenced considerably by several medium components and other factors. Rooting efficiency enhancement was obtained by reduction of mineral salt concentration by half or even more (mainly macro-elements, especially ammonium nitrate) [Sriskandarajah et al. 1990, Druart 1997] as well as medium supplementation with polyamines [Naija et al. 2009], activated charcoal [Magyar-Tábori et al. 2001a] and phenolic compounds [De Klerk et al. 2011]. Zimmerman and Fordham [1985] proved that increased temperature to even 30°C and darkness during the induction phase lasting 3–7 days significantly enhanced shoot rooting frequency. The aim of the study was to develop an effective method of rooting microcuttings of apple tetraploids newly obtained by *in vitro* technique [Podwyszyńska et al. 2017]. Due to the limited amount of tetraploid shoots, in the first stage of this research, the method of *in vitro* rooting was optimized for shoots of diploid donor cultivars. Subsequently, shoot rooting ability was compared between donor cultivars and their neo-tetraploids using the selected medium considered optimal for diploid counterparts.

MATERIAL AND METHODS

Shoot multiplication

Five scion cultivars of apple (*Malus* × *domestica* Borkh.) were used for the study: 'Free Redstar', 'Gala Must', 'Pinova', 'Redchief' and 'Sander'. *In vitro* shoot cultures were established and multiplied by axillary shoot growth stimulation. Shoots were established and continuously multiplied *in vitro* at 4–5 week subculture periods in 330 jars containing 40 ml of standard multiplication medium including modified Murashige and Skoog [1962] (MS) macro- and microelements in which iron source was changed for chelate EDDHA 80 mg l^{-1}, 3% sucrose, 100 mg l^{-1} inositol, thiamine, nicotinic acid and pyridoxine each at 1 mg l^{-1}, supplemented with growth regulators: 4.0 µM benzyladenine (BA), 0.3 µM gibberellic acid (GA_3) and 0.5 µM IBA; pH 5.6 and solidified with 6 gl^{-1} agar (Plant Propagation Labagar, Biocorp, Poland). Since in one experiment, the post effects of different cytokine types on rooting ability were tested, in the last multiplication subculture, besides BA, also *meta*-Topolin (*m*T) was used, each at the concentration of 2, 4 and 8 µM.

Tetraploids of above mentioned cultivars were obtained in our previous study using *in vitro* techniques [Podwyszyńska et al. 2017]. Selected tetraploids (two of each cultivar) were cloned to about 30 shoot clumps and such shoot cultures were maintained and served to rooting experiments. Tetraploid shoots were subcultured in the same way as their diploid counterparts.

Shoot multiplication and rooting, except when stated, were maintained at the temperature of 21°C under standard 16/8 h photoperiod of 30 µmol m^{-2} s^{-1} photosynthetic photon flux density (warm white fluorescent lamps).

Rooting

For rooting experiments, shoots 1.5–2 cm in length, derived from 16–24-month-old cultures were taken at the 5th week of subculture. The shoots were maintained on rooting induction medium (RIM) continuously for 25 days (one-step rooting system) or in some experiments, for 7 days followed by shoot transplanting onto a root elongation medium (REM) for 18 days (two-step rooting method). RIM contained ingredients of MS basic medium excluding adenine sulphate and NH_4NO_3 (in order to lower nitrate level) and supplemented with auxins (IBA or

NAA) alone or in combination with IAA and polyamine (putrescine, Put) or precursors of their biosynthesis, amino acids: arginine (Arg) and ornithine (Orn). Concentrations and combinations of auxins, polyamine and amino acids are given at particular experiment description. REM consisted of the same basic ingredients as RIM but did not contain any auxins, Put and amino acids; additionally REM was supplemented with 4 g l^{-1} of activated charcoal. After 25 days of *in vitro* rooting, rooting percentage, root number per rooted shoot and root length were evaluated. Subsequently, rooted shoots of the good quality (without leaf senescence symptoms) were planted in paper pots of 30 mm diameter, with peat-coconut substrate (Ceres International, Poland), in plastic mini-greenhouses (58 cm long × 40 cm wide × 22 cm high; XL High Dome propagator, Garland Products Ltd., UK) with 2 adjustable 'dial' ventilators to control the humidity and optimise growing conditions. Plants in mini-greenhouses were maintained for four weeks in growing chamber under fluorescent lamps (40 µmol m^2 s^{-1}) at 25°C then transferred to greenhouse for further growing. Plants were watered and high humidity was reduced gradually by opening ventilators. Percentage of plant survival was evaluated after six-week *ex vitro* growing.

Six experiments were performed in order to optimize a rooting method for apple microcuttings of the diploid donor cultivars. In the last experiment, rooting capacity was compared between donor cultivars and their neo-tetraploids using the selected medium considered optimal for donor cultivars. From one to four varieties were used in subsequent experiments depending on the availability of plant material and research goals.

Experiment 1. The effect of IBA alone (2.5, 5 and 7.5 µM) used continuously (one-step rooting) was studied on shoot rhizogenic capacity of four cultivars 'Redchief', 'Free Redstar', 'Pinova' and 'Sander'.

Experiment 2. Effects of IBA alone at 2.5 µM or combined with 5 µM IAA and/or Put (50 µM), Arg (250 µM) and Orn (250 µM) were determined in order to enhance shoot rooting efficiency of 'Redchief' (Exp. 2.1). Subsequently, three selected treatments were applied to shoot rooting evaluation of 'Free Redstar', 'Pinova' and 'Sander' (Exp. 2.2). One-step rooting method was used. Put and amino acids concentrations were selected based on preliminary study in which the positive effects of these compounds used at higher concentrations (Put at 100 µM; Arg and Orn at 500 µM) were less prominent.

Experiment 3. Shoots of 'Gala Must' were used in order to evaluate rooting efficiency in one- and two-step rooting systems. Based on earlier experiments, the selected RIMs were used which were considered the most efficient for rooting shoots of other cultivars. The media contained 2.5 µM IBA combined with 5 µM IAA and 50 µM Put or 250 µM Arg.

Experiment 4. In two-step rooting method, effects of temperature (21°C and 26°C) and 16 h photoperiod or darkness during 7-day induction phase were tested on rooting efficiency of difficult-to-root 'Free Redstar' and 'Pinova'. RIM with 2.5 µM IBA, 5 µM IAA and 50 µM Put was used. At the root elongation phase, shoots were maintained at 21°C and standard photoperiod.

Experiment 5. The effects of BA and *m*T used in the last multiplication subculture were evaluated on quality of shoots and their rooting ability. Three cultivars ('Free Redstar', 'Gala Must' and 'Redchief') were used. Two-step rooting system was utilized with darkness and temperature of 26°C during 7-day induction phase, and RIM as in experiment 4.

Experiment 6. The difficult-to-root cultivars 'Free Redstar' and 'Pinova' were tested for rooting efficiency using RIM containing NAA (1.3, 2.6 and 5.2 µM) alone or combined with 5 µM IAA in two-step method and physical conditions as in experiment 5.

Experiment 7. Rooting ability was compared between diploid cultivars and resulted neo-tetraploids of the cultivars 'Free Redstar', 'Gala Must', 'Pinova' and 'Redchief'. The two-step-rooting method was utilized as in experiment 5.

Statistical analysis

For multiplication and rooting experiments, 25–30 shoots of diploid scion cultivars (five shoots × five or six flasks) were used in each treatment. For rooting experiment with tetraploids, 16 shoots (four shoots × four flasks) were used in each treatment. Data were subjected to a one-factor (experiment No. 2.1, 5 and 6) or two factor (experiments No. 1, 2.2, 3, 4 and 7) analyses of variance ANOVA. Probability values (*P*) of

F test from ANOVA for data of each experiment were presented in corresponding tables and figures. Means were compared by HSD Tukey's test or Duncan's multiple range tests. All calculations were done with the STATISTICA package (StatSoft v. 13).

RESULTS

In general, first roots, irrespectively of the culture conditions and genotype were visible after 10–14 days.

Experiment 1. The shoots of the studied cultivars characterized with low rooting ability. In three of four studied cultivars, the shoots rooted sporadically irrespectively of IBA presence and concentration (fig. 2). In 'Redchief', the highest shoot percentage of approximately 40% was observed at IBA concentrations of 2.5 and 7.5 µM. In this cultivar, the roots produced in the presence of IBA applied continuously for 25 days were very short (2–3 mm). In greenhouse, both rooted and unrooted 'Redchief' shoots were

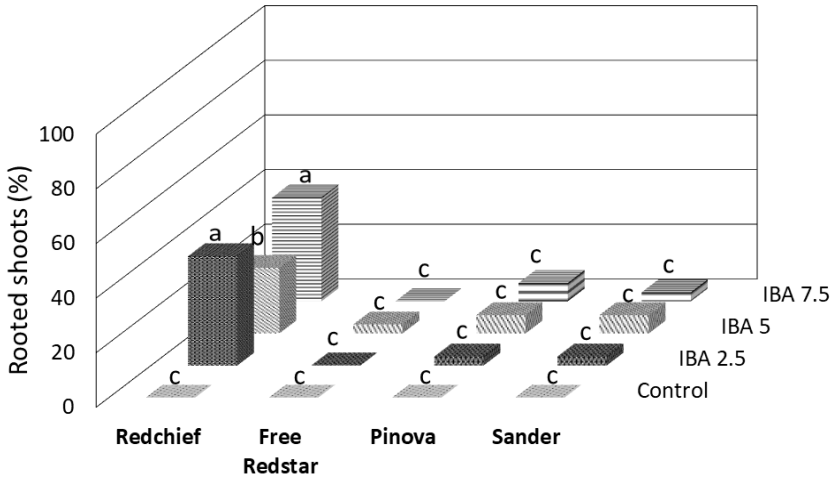

Fig. 2. The effect of IBA alone (2.5, 5 and 7.5 µM) used continuously (one-step rooting) on shoot rhizogenic capacity of four apple cultivars, 'Redchief', 'Free Redstar', 'Pinova' and 'Sander'

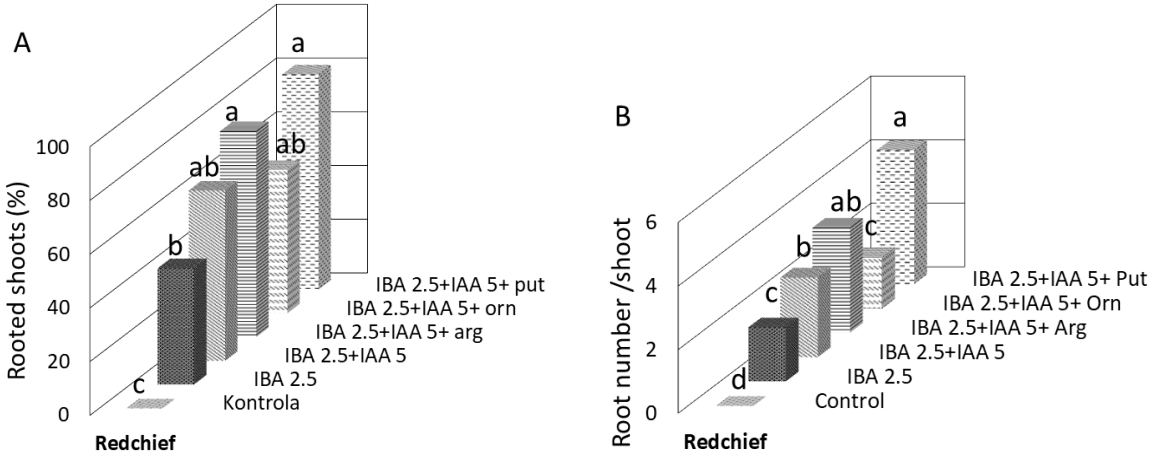

Fig. 3. Effects of IBA at 2.5 µM alone or combined with 5 µM IAA and/or Put (50 µM), Arg (250 µM) and Orn (250 µM) on shoot rooting of apple cultivar 'Redchief' using one-step rooting method

planted and only the shoots which had been rooted *in vitro* acclimatized to *ex vitro* conditions.

Experiment 2. The significant improvement of 'Redchief' shoot rooting to 63.4% was obtained due to the application of IBA at the concentration of 2.5 µM combined with 5 µM IAA (fig. 3A). The higher rooting efficiencies of 76.6% and 80% of rooted shoots were recorded when the media with this auxin combination were supplemented with 250 µM arginine or 50 µM putrescine, respectively. Moreover, in such treatments, shoots had the highest number of roots, 3.3 and 4.2, respectively (fig. 3B). Irrespectively of the treatment, the roots were very short, the average root length did not exceed 2 mm. Ornithine addition did not influence rooting. The medium containing above mentioned auxin combination and Put was also the most efficient for rooting shoots of 'Sander' (52.2%) (fig. 4). Rooting was not improved for 'Free Redstar' and 'Pinova'.

Experiment 3. Application of two-step rooting method significantly improved rooting efficiency of 'Gala Must' shoots compared to one-step rooting system. In the presence of IBA combined with IAA, the rooting percentage was two times higher (30%) in two-step method compared to one-step method (16.6%) (tab. 1). The highest rooting efficiency of 57% was observed at two-step system when the auxin combination was supplemented with 50 µM Put. Roots were significantly shorter in the presence of Put (tab. 1, fig. 1B).

Experiment 4. Temperature and 16 h photoperiod or darkness during seven-day induction phase significantly influenced rooting efficiency of the difficult-to-root cultivars 'Free Redstar' and 'Pinova'. When the seven-day root induction (using RIM with 2.5 µM IBA + 5 µM IAA and 50 µM Put) was performed in darkness, significant increase up to 77.5% in rooting percentages was observed for 'Pinova', irrespectively of temperature, whereas light reduced rooting to 48,3% and 57.5% at the temperatures of 21°C and 26°C, respectively (tab. 2). In 'Free Redstar', rooting percentage of 48% was the highest in darkness at 26°C. Light totally inhibited root formation in this cultivar.

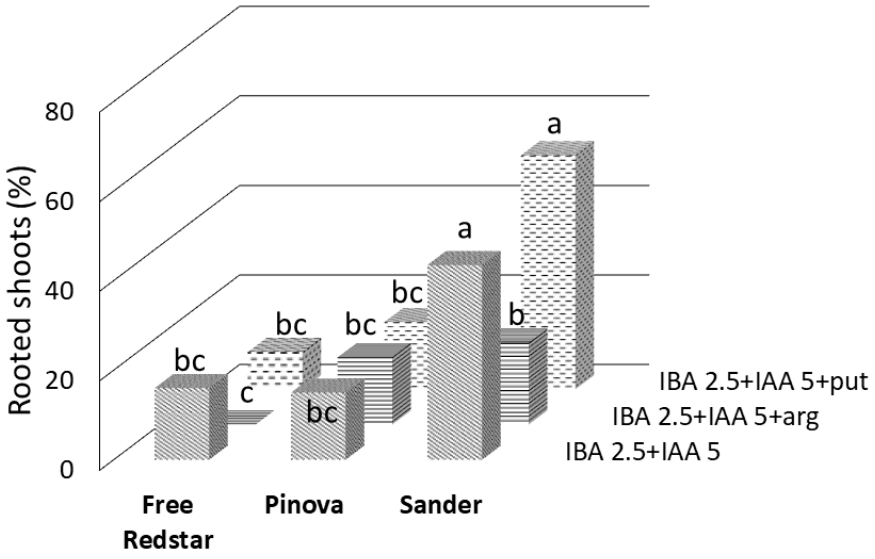

Fig. 4. Effects of IBA at 2.5 µM alone or combined with 5 µM IAA and/or Put (50 µM) and Arg (250 µM on shoot rooting of apple cultivars, 'Free Redstar', 'Pinova' and 'Sander' using one-step rooting method

Table 1. Effect of medium components (auxins, arginine and putrescine) in one- and two-step *in vitro* rooting systems on the shoot rooting efficiency of apple 'Gala Must'

Medium compounds: auxins, arginine, putrescin (μM)	Rooted shoots (%)		Root number per shoot		Root length (mm)	
	Rooting system					
	one-step	two-step	one-step	two-step	one-step	two-step
IBA 2.5 + IAA 5	16.6 b	30.0 b	–	2.9 a	–	20.3 a
IBA 2.5 + IAA 5 + Arg 250	6.7 b	28.0 b	–	2.1 a	–	18.6 a
IBA 2.5 + IAA 5 + Put 50	16.7 b	57.1 a	–	2.9 a	–	7.3 b
			P			
Medium compounds	0.00		–	0.67	–	0.01
Rooting system	0.01		–	–	–	–
Compounds × rooting system	0.08		–	–	–	–

Means separation by HSD Tukey's test; two-factor ANOVA for rooting percentage and one-factor ANOVA for root number per shoot and root length at two-step rooting system due to the insufficient data number. The means followed by the same letter do not differ significantly at $P \leq 0.05$

Table 2. Effect of temperature (21°C and 26°C) and 16 h photoperiod or darkness during 7-day induction phase on rooting efficiency of the difficult-to-root apple cultivars 'Free Redstar' and 'Pinova'; two-step rooting method using RIM with 2.5 μM IBA, 5 μM IAA and 50 μM Put

Physical factors	Rooted shoots (%)	Root number per shoot	Root length (mm)
	'Free Redstar'		
16 h photoperiod / 21°C	0.0 c	0.0 b	0.0 b
16 h photoperiod / 26°C	0.0 c	0.0 b	0.0 b
Darkness / 21°C	21.7 b	0.7 a	10.9 ab
Darkness / 26°C	48.0 a	1.2 a	15.0 a
	P		
16 h photoperiod/darkness	0.00	0.00	0.00
Temperature	0.02	0.24	0.46
16 h photoperiod/darkness × temperature	0.02	0.24	0.46
	'Pinova'		
16 h photoperiod / 21°C	48.3 b	1.2 a	1.8 ab
16 h photoperiod / 26°C	57.5 b	1.2 a	1.0 b
Darkness / 21°C	77.5 a	1.4 a	1.2 ab
Darkness / 26°C	77.5 a	2.4 a	1.9 a
	P		
16 h photoperiod/darkness	0.01	0.08	0.36
Temperature	0.54	0.18	0.76
16 h photoperiod/darkness × temperature	0.54	0.21	0.00

Means separation by HSD Tukey's test; two-factor ANOVA separately for each cultivar. The means followed by the same letter do not differ significantly at $P \leq 0.05$

Experiment 5. Cytokinins BA and *m*T used in the last multiplication subculture considerably influenced shoot quality and had post-effect on rooting ability. The higher were the cytokinin levels, the larger were the multiplication rates (tab. 3). In all the cultivars, the grater multiplication rates were recorded at the highest BA concentration of 8 µM, and were generally significantly different from other treatments. However, the longest shoots of all the cultivars were obtained at *m*T concentrations of 4 µM and/or 8µM. Surprisingly, the application of BA during the last multiplication subculture at the higher concentration of 4 and 8 µM as well as *m*T at 4 µM increased the rooting efficiency compared to cytokinin treatment with lower concentrations (tab. 3). The highest rooting percentage of 76% in 'Free Redstar' was recorded for 4 µM *m*T pretreatment and this result did not differ significantly from 4 and 8 µM BA pre-

Table 3. Effect of BA and m-T on shoot multiplication and quality and post-effect of these cytokinins on rooting *in vitro* and acclimatization of micropropagated apple scion cultivars; two-step rooting, using RIM with 5 µM IBA, 5 µM IAA and 50 µM Put and darkness and 26°C during 7-day induction phase

Cultivar, cytokinin (µM)	Shoot number	Shoot length (mm)	Rooted shoots (%)	Root number per shoot	Root length (mm)	Acclimatized shoots per planted shoots (%)
			'Free Redstar'			
BA 2	2.9 c	23.6 b	50.0 b	3.1 ab	15.6 a	8/10 (80.0)
BA 4	6.4 b	28.9 ab	56.0 ab	2.4 ab	13.2 a	10/16 (62.5)
BA 8	9.5 a	26.9 ab	72.0 ab	3.8 a	15.7 a	8/19 (44.6)
mT 2	1.9 c	28.2 ab	48.0 b	1.6 b	11.1 a	14/16 (87.5)
mT 4	3.8 bc	35.3 a	76.0 a	2.4 ab	8.3 a	13/18 (72.2)
mT 8	6.5 b	23.1 b	52.0 b	1.6 b	15.7 a	10/14 (71.1)
P	0.00	0.20	0.04	0.04	0.21	
			'Gala Must'			
BA 2	3.0 b	38.4 b	60.0 abc	2.5 a	27.6 a	12/12 (100.0)
BA 4	5.1 a	38.1 b	85.0 a	2.1 ab	16.2 c	17/17 (100.0)
BA 8	6.4 a	33.2 b	80.0 ab	2.5 a	16.3 bc	5/8 (62.5)
mT 2	1.5 b	37.2 b	57.9 bc	1.1 b	18.0 b	17/17 (100.0)
mT 4	2.2 b	47.1 a	50.0 c	1.5 ab	10.0 cd	14/15 (93.3)
mT 8	6.2 a	46.2 a	60.0 abc	1.1 b	6.0 d	12/14 (85.7)
P	0.00	0.00	0.07	0.06	0.00	
			'Redchief'			
BA 2	2.5 cd	46.7 b	70.0 ab	1.9 bc	20.1 a	12/13 (93.3)
BA 4	3.8 bc	42.5 b	75.0 ab	3.3 abc	17.8 ab	15/16 (93.4)
BA 8	5.9 a	42.6 b	90.0 a	5.0 a	15.3 abc	11/16 (68.8)
mT 2	1.9 d	57.6 a	60.0 b	1.1 c	12.2 bcd	9/11 (81.8)
mT 4	5.0 ab	56.9 a	90.0 a	3.9 ab	11.1 cd	16/16 (100.0)
mT 8	4.5 ab	64.4 a	70.0 ab	1.30 c	7.9 d	13/14 (92.9)
P	0.00	0.00	0.08	0.01	0.00	

Means separation within columns by Duncan's multiple range test; one-factor ANOVA separately for each cultivar. The means followed by the same letter do not differ significantly at $P \leq 0.05$

treatments (56% and 68%, respectively). Similarly in 'Gala Must' and 'Redchief', the best rooting efficiencies of 80% to 90% were obtained at the pretreatments of 4 and 8 µM BA or 4 µM *m*T. The root numbers per shoots were also higher for such pretreatments. Roots were generally longer when BA was used during shoot multiplication stage compared to *m*T applications. Roots were the shorter, the higher were the cytokinin levels. BA at the highest concentration, however, reduced markedly plants survival while *m*T did not affect negatively the acclimatization capacity (tab. 3).

Experiment 6. NAA applied in two-step rooting method (with darkness and 26°C at 7-day induction phase) strongly stimulated rhizogenesis of shoots of the difficult-to-root cultivars, 'Free Redstar' and 'Pinova' (tab. 4). In 'Free Redstar', NAA at 2.6 and 5.2 µM combined with 5 µM IAA increased rooting percentage to approximately 70 and 55%, respectively whereas only 8% of shoots formed roots in control (the auxin-free medium). At 2.6 µM NAA treatments the highest rate of plants were successfully acclimatized to *ex vitro* conditions. In 'Pinova', NAA alone at 1.3 and 2.6 µM increased markedly rooting efficiency to over 80% compared to control in which only 4.8% shoot were rooted. In 'Pinova', plant acclimatization to *ex vitro* conditions was very poor, except for treatments by NAA 2.6+IAA 5 and NAA 5.2+IAA 5, only a single plant per treatment survived in a greenhouse irrespectively of the auxin treatment. The longest roots of both cultivars were observed at the lowest auxin concentration (1.3 µM NAA alone). NAA at highest concentration of 5.2 µM strongly inhibited rooting.

Table 4. Effect of auxins on rooting *in vitro* shoots of apple cultivars 'Free Redstar' and 'Pinova'; two-step rooting sysytem with darkness and 26°C at 7-day rhizogenesis induction

Auxin concentration (µM)	Rooted shoots (%)	Root number per shoot	Root length (mm)	Acclimatized shoots per planted shoots (%)
		'Free Redstar'		
Control	8.0 c	–	–	2/2
NAA 1.3	12.0 c	1.0 a	2.7 a	1/3
NAA 2.6	40.0 b	1.5 ab	2.0 a	8/10
NAA 5.2	16.0 c	1.9 ab	1.6 a	4/4
NAA 1.3 + IAA 5	70.0 a	2.7 a	1.9 a	6/15
NAA 2.6 + IAA 5	55.0 ab	2.8 a	2.0 a	2/7
NAA 5.2 + IAA 5	4.0 c	–	–	2/2
P	0.00	0.045	0.81	
		'Pinova'		
Control	4.8 b	–	–	1/1
NAA 1.3	81.8 a	4.2 a	3.8 a	1/18
NAA 2.6	84.4 a	3.0 ab	1.4 b	1/19
NAA 5.2	68.2 ab	2.7 ab	1.6 b	1/15
NAA 1.3 + IAA 5	85.7 a	3.5 ab	2.7 ab	1/18
NAA 2.6 + IAA 5	59.1 ab	3.0 ab	1.9 ab	2/13
NAA 5.2 + IAA 5	59.1 ab	1.7 b	1.2 b	4/13
P	0.00			

Means separation within columns by HSD Tukey's test; one-factor ANOVA separately for each cultivar. The means followed by the same letter do not differ significantly at $P \leq 0.05$

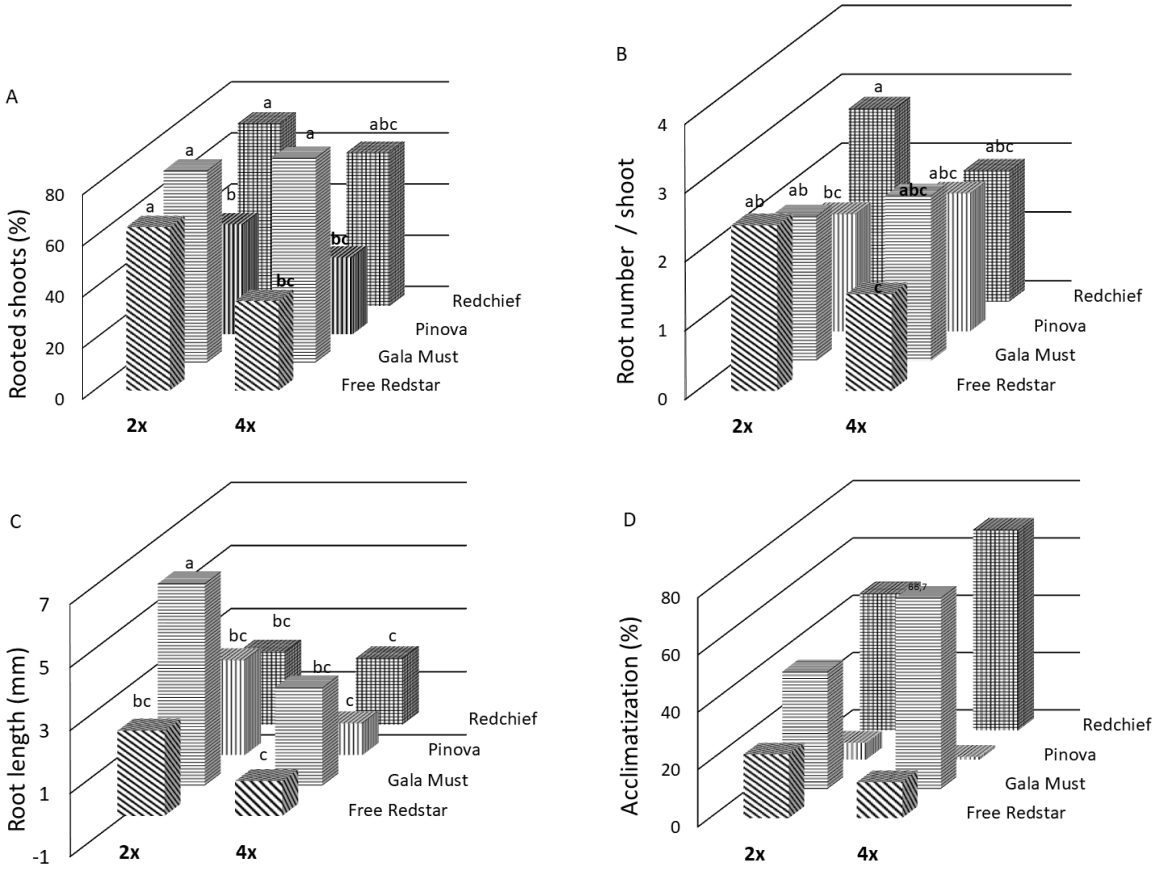

Fig. 5. Evaluation of rooting ability of diploid donor cultivars ('Free Redstar', 'Gala Must', 'Pinova' and 'Redchief') and their neo-tetraploids; the two-step-rooting method darkness and temperature of 26°C during 7-day induction phase; RIM contained 2.5 µM IBA combined with 5 µM IAA and 50 µM Put

Experiment 7. Shoot rooting efficiency was compared between diploid donor cultivars ('Free Redstar', 'Gala Must', 'Pinova' and 'Redchief') and the resulted neo-tetraploids. Shoot rhizogenic abilities of particular tetraploids generally reflected the abilities of their diploid counterparts. Tetraploids of 'Free Redstar' and 'Pinova' characterized with the lowest rooting percentages, 35.0% and 30%, respectively, compared to their diploid counterparts, 64.3% and 42.9%, respectively as well as to remaining diploid and tetraploid genotypes (fig. 5A). In other cultivars, rooting efficiencies of tetraploid and diploid plants were comparable, 'Gala Must' 80.0% and 79.5%, respectively, and 'Redchief' 60.0% and 85.7%, respectively. Generally, in tetraploids, the lower root number per shoot was recorded and the roots were shorter (fig. 5B, C). In 'Gala Must' and 'Redchief' acclimatization efficiency was higher for tetraploids (fig. 1C, 1D, 5D). After 8 weeks of *ex vitro* growing in a greenhouse, phenotypic differences between the diploid and tetraploid plants were clearly visible (fig. 1E, F). The tetraploids had shorter shoots with darker green leaves compared to diploids.

DISCUSSION

Our results showed that except for 'Redchief' with 40% shoot rooting rate, the microcuttings of

other cultivars produced roots sporadically, despite of the application of the rooting media with lowered nitrate level and IBA (2.5–10 µM) supplementation, recommended by several authors as reviewed by Dobránszki and Teixeira da Silva [2010]. Apple genotypes used in our experiments are the scion cultivars of which shoots propagated *in vitro* are considered difficult-to-root [Lane and McDougald 1982].

Considerable increase in shoot rooting efficiency of the cultivar 'Redchief' to 63.4% was obtained due to the use of IBA and IAA combination. Similarly, Druart [1997], using IBA (1 mg l^{-1}) combined with IAA (0.1 mg l^{-1}) obtained 100% rooting of the cultivar 'Compact Spartan'. The successful rooting could be due to the specific effect of IBA which was found to increase the level of endogenous free IAA [Nag et al. 2001]. Besides, it is demonstrated that rhizogenesis is induced only by free auxins [De Klerk et al. 1999, Nag et al. 2001]. Moreover, IBA is considered IAA precursor since in several plants auxin activity of IBA is completely dependent on its conversion to IAA by peroxisomal enzymes [Strader and Bartel 2011]. Strong rhizogenic activity of IBA is ascribed to its higher stability in tissue. Namely, IBA conjugates are not oxidized in contrast to IAA which conjugates are quickly degraded [Van Der Krieken et al. 1992, Epstein and Ludwig-Müller 1993].

Although IBA was used most frequently for root induction in apple [Dobránszki and Teixeira da Silva 2010], NAA (0.3 mg l^{-1}) was proved to be very effective in rooting shoots of several scion apple cultivars [Zimmerman and Fordham 1985]. We also found that 1.3 µM NAA alone or combined with 5 µM IAA, applied during 7-day induction phase, significantly enhanced rooting efficiency of the difficult-to-root cultivars, 'Free Redstar' and 'Pinova'. Similar synergistic effect of auxin combinations (IBA+IAA or NAA+IAA) on rooting was reported for rooting of *Pongamia pinnata* cuttings [Kesari et al. 2009].

The differences in rooting activity of various auxins result from their various uptake, metabolism and transport [Van der Krieken et al. 1992, Epstein and Ludwig-Müller 1993, Strader and Bartel 2011]. NAA is taken up by shoots much faster than IAA [Peeters et al. 1991]. On the other hand, studies of auxin transport show that IAA is transported slightly faster than NAA and much faster than IBA [Epstein and Ludwig-Müller 1993]. NAA exists mostly in the conjugated form and has minor effects on endogenous IAA level [Smulders et al. 1990]. IAA is both conjugated and oxidized [Epstein and Ludwig-Müller et al. 1993].

In our study, significant improvement of rooting efficiency was achieved by addition of putrescine to RIM containing auxin combination. Similar polyamine effect on root formation of apple rootstock microcuttings was reported by Naija et al. [2009]. In turn, the high positive correlation between *in vitro* rooting and the increased level of endogenous free polyamines, particularly Put was reported by Neves et al. [2002]. Moreover, these authors concluded that free Put can be considered as a marker of *in vitro* root induction phase. Nag et al. [2001] suggested that treatment with IBA reduces IAA-oxidase activity, and thus increased endogenous IAA level at very early rhizogenesis induction phase. Both IBA and Put enhanced at later phase peroxidase activity which is involved in cell wall formation, related to root initiation. All these data indicate that IAA and Put play in common an important role in rhizogenesis.

In our study, addition of arginine, but not ornithine improved *in vitro* rooting of apple shoots. Beneficial effect of arginine on shoot rooting of apple dwarf rootstock *in vitro* was also reported by Orlikowska [1992a,b]. Furthermore, the ornithine effect was shown to be less prominent. These amino acids are precursors of putrescine biosynthesis [Bais and Ravishankar 2002]. Put may be formed directly, through ornithine decarboxylation, or indirectly, through a series of intermediates, following arginine decarboxylation. The positive impact of arginine on rooting, observed in our and other studies may indicate major role for arginine decarboxylation rather than ornithine in Put synthesis. It was suggested that such a mechanism of Put biosynthesis with arginine decarboxylation could function in *Phaseolus* [Palavan-Ünsal 1987] and cork oat [Neves et al. 2002].

Considerable increase of rooting efficiency to 57%, shown in 'Gala Must', was obtained by short auxin treatment (7 days) in two-step rooting method compared to continuous auxin application (16.6%). It was well documented that auxins are only needed

during the induction of root formation [De Klerk et al. 1999, Nag et al. 2001].

Among other factors strongly influencing root formation, elevated temperature to even 30°C and darkness during an induction phase are reported to improve rooting efficiency of apple microcuttings [Zimmerman and Fordham 1985, Orlikowska 1992b]. Our results supported these findings, since initial darkness in common with increased temperature from 21°C to 26°C was prerequisite for rooting shoots of difficult-to-root 'Free Redstar' and darkness enhanced by 50% rooting efficiency in another recalcitrant cultivar 'Pinova'.

Lowering cytokinin level in the last multiplication subculture before rooting improved rhizogenic capacity of microcuttings as it was reported for apple [Dobránszki et al. 2000] and smoke bush [Podwyszyńska et al. 2012]. It was also shown that another naturally occurring aromatic cytokinin, *m*T, due to its faster degradation in plant tissue, did not have negative effect on rhizogenic capacity [Werbrouck et al. 1995, 1996, Wojtania et al. 2011]. In our study, however, neither lowering the cytokinin concentration nor replacement of BA to *m*T, which is BA derivative, positively influenced rooting. Suprisingly, BA at higher concentrations (4 and 8 µM) enhanced rhizogenic capacity. Contrary response of apple microcuttings to *m*T and cytokinin higher levels were reported by Dobránszki et al. [2000] and Magyar-Tábori et al. [2001b]. On the other hand, *m*T positively influenced acclimatization capacity in contrast to BA which at higher concentration reduced percentage of plant survival in *ex vitro* conditions.

Our results showed that tetraploids characterized by comparable or slightly different shoot ability to rooting and acclimatization in relation to their diploid counterparts. Differences in phenotype and *in vitro* growth behaviour between diploids and their neotetraploids were reported for several plant species, e.g. watermelon [Compton et al. 1993], gerbera [Gantait et al. 2011] and pear [Sun et al. 2011]. The above-mentioned authors also observed that *in vitro* produced neo-tetraploid shoots are generally shorted and have wider leaves darker colour compared to diploid ones. Shoot regeneration potential from leaves in pear [Gantait et al. 2011] and shoot multiplication rate in gerbera [Sun et al. 2011] were significantly lower in tetraploids compared to diploids. In gerbera, shoots of tetraploids required longer period to produced roots and the roots were shorter and less numerous compared to those of diploids. In contrast, shoot rooting efficiency of watermelon [Compton et al. 1993] was similar in diploid and tetraploid plants. In pear, rhizogenic ability was highly dependent on genotype and a ploidy level had minor effect on rooting. These observations correspond with ours, since we found comparable rooting and acclimatization ability in diploid and tetraploid plants of particular apple cultivars. Significant differences in rhizogenic ability within the donor cultivar group and the tetraploid group were generally analogical.

Summarizing, tetraploids of four apple cultivars derived by *in vitro* technique were successfully rooted *in vitro* and acclimatized to *ex vitro* conditions. The tetraploid plants after phenotypic and genetic evaluation will be used for further breeding works.

CONCLUSIONS

1. Shoot of all the studied apple scion cultivars have low rooting ability.

2. Considerable improvement of apple shoot rooting to 60–80% can be achieved through the application of auxins, 2.5 µM IBA or 1.3 µM NAA combined with 5 µM IAA and putrescine in the two-step rooting system with darkness and enhanced temperature (26°C) during seven-day induction phase.

3. BA at highest concentration reduced markedly number of acclimatized plants while *m*T, irrespectively of concentration did not affect acclimatization frequency.

4. In the cultivars with low rhizogenic capacity, satisfactory rooting efficiency can be obtained through the use of NAA in the root-induction medium.

5. Tetraploids characterized by comparable or slightly lower ability to rooting compared to their diploid counterparts.

6. The optimized method of rooting *in vitro* ensure successful acclimatization of neo-tetraploids to *ex vitro* conditions

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