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EFFECTS OF PLANT GROWTH REGULATORS AND ENVIRONMENTAL FACTORS ON *IN VITRO* PROPAGATION OF ×*MALOSORBUS FLORENTINA*

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Abstract

Aim of the work was to improve the micropropagation protocol of the rare and endangered ×*Malosorbus florentina* enabling its conservation and introduction as an ornamental plant. The micropropagation of the species is difficult at the establishment and rooting phase. Using of thidiazuron (TDZ) instead of BA or zeatin at the culture initiation phase almost doubled the establishment percentage of the explants. The shoots produced on a TDZ-supplemented MS medium were short, but following subculture on a MS medium supplemented with 1 mg l^{-1} BA + 0.1 mg l^{-1} IBA explants from TDZ-medium surpassed those from BA- or zeatin-medium in shoot number without falling short in shoot length. BA at 1-2 mg l^{-1} proved superior to zeatin, kinetin, and 2iP in multiplication. Darkness during the root induction phase (one week on auxin supplemented half-strength MS medium) did not improve rooting and stimulated excessive callus formation. Activated charcoal (AC) enhanced rooting of juvenile origin microshoots when added into the medium during the first week of the root expression phase (four weeks on hormone free half-strength MS medium). Microshoots produced on a medium with low cytokinin concentration or on a BA-medium rooted at higher percentages than those produced on a high-cytokinin medium or on a zeatin- or 2iP-medium. Higher rooting percentage (65%) was induced on half-strength MS medium with 4 or 8 mg l^{-1} IAA. Higher acclimatization percentages and faster growth were achieved when plantlets with well developed roots were transferred *ex vitro* on a peat-perlite (1 : 1, v/v) mixture in early spring.

Key words: acclimatization, activated charcoal, auxins, cytokinins, darkness, rooting

INTRODUCTION

×*Malosorbus florentina* (Zucc.) Browicz (Rosaceae) occurs in the Balkan Peninsula, Italy and Northern Turkey, and it is a rare and threatened species in Greece (Christensen 1995). It was originally described as *Crataegus florentina* by the Italian Botanist Zuccagni in 1809. It has a complex taxonomic history; since it was described, it has been referred to *Pyrus*, *Mespilus*, *Torminaria*, *Sorbus*, *Cormus*, as well as *Malus* and *Eriolobus* and the latest years to the monotypic genus ×*Malosorbus florentina* (*Malus* × *Sorbus*) (Browicz 1970, 1983, Cheng et al. 2000, Christensen 1995). This plants has received considerable interest by researchers studying the very complex evolution of the maloids (Cheng et al. 2000, Samorodova-Bianki et al. 1990), as well as the pathogen resistance of wild *Malus* species (Lee et al. 2000, Schuster et al. 2000). It is considered a natural intergeneric hybrid of *Malus pumila* and *Sorbus torminalis* (Tutin and al. 1968) or *Malus sylvestris* and *Sorbus torminalis* (Browicz 1970, 1983, Samrodova-Bianki et al. 1990), although a genetic study showed that it is unlikely to be derived from *Malus sylvestris* and pointed out Asiatic *Malus* species as parental (Manganaris and Alston 1992). In the new system of *Malus* taxonomy, *Malosorbus florentina* is recognized as an independent section with one species (Cheng et al. 2000).

The plant has attractive broadly ovate, toothed, deeply lobbed leaves with a dark green and glabrous upper surface and grayish-green and hairy lower surface, turning orange-scarlet in autumn. It bares white flowers in corymb or umbel inflorescences in late spring, and red or yellowish-red, 8-14 mm in diameter fruits in early autumn. These features give *M. florentina* particular horticultural value (Christensen 1995) and make it suitable for use as an ornamental landscape plant, particularly in mountainous urban or suburban areas.

In order to introduce the plant in the ornamental

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horticulture industry, an efficient protocol for propagation should be developed. Personal observations from 2004 to 2011 in the population of ×*M. florentina* on Mt. Parnitha near Athens (one of the two populations of the species in Southern Greece), showed that it is difficult to find seeds because either plants do not produce fruits, or fruits produced do not contain seeds due to pollination problems related to the extremely low number of trees. *In vitro* propagation, which is increasingly used in the conservation of threatened plants in recent years (Fay 1994, Sarasan et al. 2006), may be the only option for propagation of this plant, at least at the initial stage, as other methods of vegetative propagation will put a pressure to native populations.

Previous studies showed that *in vitro* shoot multiplication of ×*M. florentina* was quite successful using Murashige and Skoog's (1962) medium (MS) supplemented with 1 mg l^{-1} N^6-benzyladenine (BA) and 0.1 mg l^{-1} indole-3-butyric acid (IBA), as cultures were maintained for almost 50 subsequent subcultures giving about 6 shoots per explant (Martini et al. 2013). Multiplication could be even more productive alternating the above medium with MS supplemented with 0.1 mg l^{-1} (1-Phenyl-3-(1,2,3,-thiadiazol-5-yl)urea (Thidiazuron, TDZ) (Martini and Papafotiou 2011). The major limiting factors in the efficiency of a micropropagation protocol of the plant are the low establishment percentage of buds from adult plants due to contamination and browning (Papafotiou and Martini 2009a,b), along with the poor rooting ability of microshoots (Martini and Papafotiou 2009). Explant origin, location and season of collection significantly affect the content of total phenolics and thus explant browning, as well as the shoot proliferation rate of established cultures, the rooting ability of excised microshoots and the morphology of the plantlets after acclimatization (Martini et al. 2013). Thus nodal explants excised during summer months from juvenile or rejuvenated stock plants were suggested for the establishment of ×*M. florentina in vitro* cultures (Martini et al. 2013), but still the culture establishment percentage remain moderate. Only the use of young seedlings as stock material induced culture establishment at high percentages (Martini et al. 2013, Savić et al. 2006), but seeds are not easily found, as mentioned above, and seedlings are of unknown genotype, making difficult the propagation of selected trees with particular characteristics, which can only be achieved through the propagation of adult plants.

Aim of this study was to improve the effectiveness of micropropagation of ×*M. florentina*, in order to enable its conservation and facilitate its use as an ornamental plant. The effect of (a) cytokinin type on *in vitro* culture establishment, (b) cytokinin type and concentration on shoot proliferation, (c) auxins, cytokinins, darkness and activated charcoal (AC) on microshoot rooting, as well as (d) the effect of plantlet quality, season, AC, and plantlet origin on acclimatization of ×*M. florentina* were investigated.

MATERIALS AND METHODS

Culture establishment experiment

The stock plants (5 or 8 months old) derived from micropropagation of a single adult ×*M. florentina* plant found on Mount Parnitha, near Athens, and they were growing in the experimental field of the Laboratory of Floriculture and Landscape Architecture, Agricultural University of Athens. Shoot tips and nodal segments, 0.8-1.0 cm long, with one (usually) or two buds, excised in July 2010 were used as explants. The explants were dipped in a water solution of ascorbic acid (AA) and citric acid (CA) (150 mg l^{-1} each) for 30 min in order to reduce explants browning, followed by surface disinfection by 30% (v/v) commercial bleach solution with a few drops of Tween 20 for 10 min. Four 3-min rinses with sterile distilled water containing AA and CA (150 mg l^{-1} each) followed.

For the induction of axillary shoots, the explants were cultured for 6 weeks on MS medium supplemented with 1.0 mg l^{-1} BA + 0.1 mg l^{-1} IBA (basal medium) or 1.0 mg l^{-1} (6-[4-Hydrooxy-3-methil-but-2-enylamino] purine (zeatin) + 0.1 mg l^{-1} BA or 1.0 mg l^{-1} TDZ, as well as MS medium without plant growth regulators (PGRs). Twenty four explants per treatment were used and the experiment was repeated twice with similar results (checked with ANOVA). The results of both experiments were pooled for the statistical analysis. The final number of explants per treatment used in the statistical analysis (*n*) depended on contamination (Table 1).

Axillary shoot multiplication experiments

Nodal segments 0.5 cm long excised from induced axillary shoots (in cultures established as described above) were cultured on the basal medium in order to investigate possible carryover effects of PGRs used at the establishment stage on shoot multiplication at a subsequent subculture. The number of explants per treatment (shown on the data relevant table) depended on the number and length of the microshoots produced at the establishment phase in each plant growth regulator combination.

In order to investigate the effectiveness of various cytokinin types on shoot multiplication, nodal segments 0.5 cm long were cultured on MS medium supplemented with 0.1 mg l^{-1} IBA and 0.5, 1.0, 2.0 or 4.0 mg l^{-1} BA, zeatin, kinetin or 2iP. The nodal segments were excised from axillary shoots derived from cultures established *in vitro* on the basal medium in June 2009, and maintained for two years with continuous subcultures on the same medium. Sprouts of wild plants burned in the 2007 fire at Mt. Parnitha were used as stock plants for those cultures,

and the method employed for their *in vitro* establishment was described by Martini et al. (2013). Thirty explants per treatment were used in two repetitions.

In both experiments explants were cultured individually in 75 ml glass vessels with 20 ml medium covered with plastic wrap. Each experiment lasted six weeks.

Rooting

Axillary microshoots for rooting experiments were excised from *in vitro* cultures established on the basal medium either from an adult plant in March 2004 or from sprouts (juvenile material) of the same plant (once it was burned) in June 2009, as described by Martini et al. (2013). Those cultures were maintained with continuous subcultures on the basal medium for almost six and two years, respectively. Microshoots 1.0-2.0 cm long were cultured on half-strength MS medium with 2% (w/v) sucrose supplemented with 0.5 mg l^{-1} IBA and 8.0 mg l^{-1} indole-3-acetic acid (IAA) for one week (root induction phase), followed by transfer on the same medium without PGRs for another four weeks (root expression phase). The culture took place under 16 h light. This rooting technique (basal rooting technique) has been found to improve rooting percentage in previous experiments (Martini 2013, Martini and Papafotiou 2009). Rooting of microshoots took place in 75 ml glass vessels covered with plastic wrap. The results were evaluated after five weeks of culture. Rooting treatments were designed in order to test the effect of various factors on microshoot rooting. Some treatments were repeated many times (1-7) during a two year period, microshoot origin and *in vitro* conditions remaining the same and the results were pooled for the statistical analysis, leading to different number of explants per treatment, which are marked on the data tables.

Light

Microshoots of adult or juvenile origin were treate d with the basal rooting technique, but during the root induction phase they were cultured either under 16 h light or in continuous darkness, followed by transfer to 16 h light during rooting expression phase. One repetition for adult and seven for juvenile origin explants were done. The results were evaluated after five weeks of culture.

Activated charcoal

Microshoots of adult or juvenile origin were cultured on half-strength MS medium with 0.5 mg l^{-1} IBA + 8.0 mg l^{-1} IAA for one week, followed by transfer to half-strength MS without PGRs for four weeks, or to half-strength MS without plant growth regulators with 2 g l^{-1} AC for four weeks, or to half-strength MS with AC for one week and then to half-strength MS for another three weeks. Three repetitions were done.

Cytokinin type in multiplication medium

Explants (nodal segments) from sprout cultures (maintained on basal medium) were subcultured once on MS medium supplemented with 0.1 mg l^{-1} IBA and 0.5-4.0 mg l^{-1} BA or 0.5-2.0 mg l^{-1} zeatin (as described above). The produced microshoots followed the basal rooting technique in order to investigate the effect of cytokinin type used for shoot multiplication on subsequent rooting of the microshoots. The results were evaluated after five weeks of culture. Three repetitions were done.

PGRs concentration in multiplication medium

Explants (nodal segments) from adult or sprout cultures (maintained on basal medium) were subcultured either on the basal medium (as routinely) or on MS medium supplemented with 0.5 mg l^{-1} BA + 0.05 mg l^{-1} IBA. The microshoots produced followed the basal rooting technique in order to investigate the effect of decreased concentrations of BA and IBA in the multiplication phase on subsequent rooting of the microshoots. The results were evaluated after five weeks of culture. Three repetitions were done.

Auxin type and concentration in rooting medium

Microshoots from sprout cultures were put on half-strength MS medium with 0.0, 0.5 or 1.0 mg l^{-1} IBA or NAA and 0.0, 4.0 or 8.0 mg l^{-1} IAA, in all possible combinations, for one week, followed by transfer on half-strength MS medium without PGRs for another four weeks in order to investigate the effect of auxin type and concentration on microshoot rooting. The results were evaluated after five weeks of culture. Two repetitions were done.

Culture conditions

All variants of the medium were solidified with 0.8% (w/v) agar (Roumboulakis S.A., Greece) and the pH was adjusted to 5.7 before autoclaving at 121°C for 20 min. Explants were cultured individually in test tubes (25 × 100 mm) covered with plastic wrap (SANITAS, Sarantis S.A., Greece). Cultures were incubated in a growth chamber at 25 ± 2°C and 16 h photoperiod at 37.5 μmol m^{-2} s^{-1} provided by cool-white fluorescent light.

Acclimatization

Plantlets were transferred *ex vitro* to 500 ml plastic containers (eight plantlets per container) on a mixture of peat and perlite (1 : 1 v/v). A sphagnum peatmoss (Highmoor) was used with a degree of humification H_3-H_5 and pH adjusted at 5.6. The perlite used was Perloflor by Isocon S.A., Greece. The containers were covered with plastic wrap in order to reduce water loss for the first seven days and placed in a growth chamber for one week at 20 ± 2ºC under a 16 h photoperiod at 37.5 μmol

$m^{-1} s^{-1}$ provided by cool-white fluorescent lamps, before being transferred to a heated glasshouse. Plantlets were watered twice a week and fertilized biweekly with 2 g l^{-1} of a complete water-soluble fertilizer (Nutrileaf 60, 20-20-20; Miller Chemical and Fertilizer Corp., Hanover, PA, USA).

Aiming to study the effectiveness of acclimatization, the following variants were distinguished: i) Plantlets derived from either adult plant or sprout (juvenile) cultures on basal medium and transferred to the greenhouse for acclimatization in January 2010 (Greenhouse T_{min} = 14°C, T_{max} = 32°C); measurements were taken after 22 weeks, ii) Plantlets derived from sprout cultures that had developed *in vitro* poor (1-3 extremely short roots, usually 0.2-0.4 cm in length) or rich (well developed roots, longer than 0.6 cm) rooting system and transferred to the greenhouse in February 2010 (Greenhouse T_{min} = 14°C, T_{max} = 32°C); measurements were taken after 22 weeks, iii) Plantlets derived from sprout cultures that were transferred to the greenhouse for acclimatization in November 2009 (Greenhouse T_{min} = 14°C, T_{max} = 39°C), January and March 2010 (Greenhouse T_{min} = 16°C, T_{max} = 32°C). Measurements were taken after 16 weeks.

Statistical analysis

The completely randomized design was used. The significance of the results was tested by one- or two-way analysis of variance (ANOVA) and the means of the treatments were compared by the Tukey-Kramer HSD at $p < 0.05$ (JMP software, SAS Institute, Cary, NC, USA). The standard error (SE) of the mean of each treatment was calculated.

RESULTS AND DISCUSSION

Culture establishment

As ×*M. florentina* is a rare and endangered plant in Greece no extended experiments concerning the establishment stage of *in vitro* cultures are possible. Previous work indicated that the establishment of cultures from either adult or rejuvenated stock plants was lower than 36% and results were obtained on MS supplemented with 1 mg l^{-1} BA and 0.1 mg l^{-1} IBA, which we now use as basal medium (Martini et al. 2013, Papafotiou and Martini 2009a,b). IBA was added to the basal medium because it enhanced shoot length at the establishment stage, as well as shoot multiplication (Papafotiou and Martini 2009a,b, Martini and Papafotiou in press).

The replacement of BA by zeatin in the basal medium did not differ from the control, while the use of TDZ instead of BA + IBA induced a higher explant response (Table 1, Fig. 1A,B,C,D). In the present work TDZ was used without IBA in the culture establishment medium, because in previous work on ×*M. florentina* the addition of IBA in an MS medium supplemented with TDZ did not affect the number and length of microshoots produced at the multiplication stage, though it increased callus formation (Martini and Papafotiou 2011). TDZ effect could be attributed to its property of mimicking both auxin and cytokinin effects, although, chemically, it is totally different from commonly used auxins and cytokinins (Murthy et al. 1998, Guo et al. 2011), as well as to its characterization as the most active cytokinin-like substance for woody plant tissue culture (Huetteman and Preece 1993, Khurana et al. 2005). Although the mode of TDZ action is unknown,

Table 1. Effect of PGRs used on explant response at the A) establishment stage and B) their carry over effect at the first subculture.

A) Establishment stage				
Variants of the medium (mg l^{-1})	Bud sprouting (%)	Shoot number	Shoot length (cm)	Explant number
MS	29.4 ± 4.3 b	1.0 ± 0.0 b	0.6 ± 0.1 b	*n* = 31
MS + 1.0 BA + 0.1 IBA	28.9 ± 4.1 b	3.1 ± 0.6 a	1.2 ± 0.3 a	*n* = 38
MS + 1.0 zeatin + 0.1 IBA	31.9 ± 4.2 b	1.0 ± 0.0 b	1.3 ± 0.2 a	*n* = 37
MS + 1.0 TDZ	53.8 ± 3.2 a	3.5 ± 0.4 a	0.6 ± 0.1 b	*n* = 39
B) First subculture				
Variants of the medium (mg l^{-1})	Shooting (%)	Shoot number	Shoot length (cm)	Explant number
MS	100.0 ± 0.0 a	1.9 ± 0.5 b	1.6 ± 0.2 a	*n* = 10
MS + 1.0 BA + 0.1 IBA	100.0 ± 0.0 a	2.6 ± 0.2 b	1.0 ± 0.1 b	*n* = 56
MS + 1.0 zeatin + 0.1 IBA	100.0 ± 0.0 a	2.6 ± 0.4 b	1.4 ± 0.1 a	*n* = 21
MS + 1.0 TDZ	100.0 ± 0.0 a	4.0 ± 0.2 a	1.2 ± 0.1 ab	*n* = 65

Means ± SE within a column followed by the same letter are not significantly different according Tukey-Kramer HSD test at $p < 0.05$.

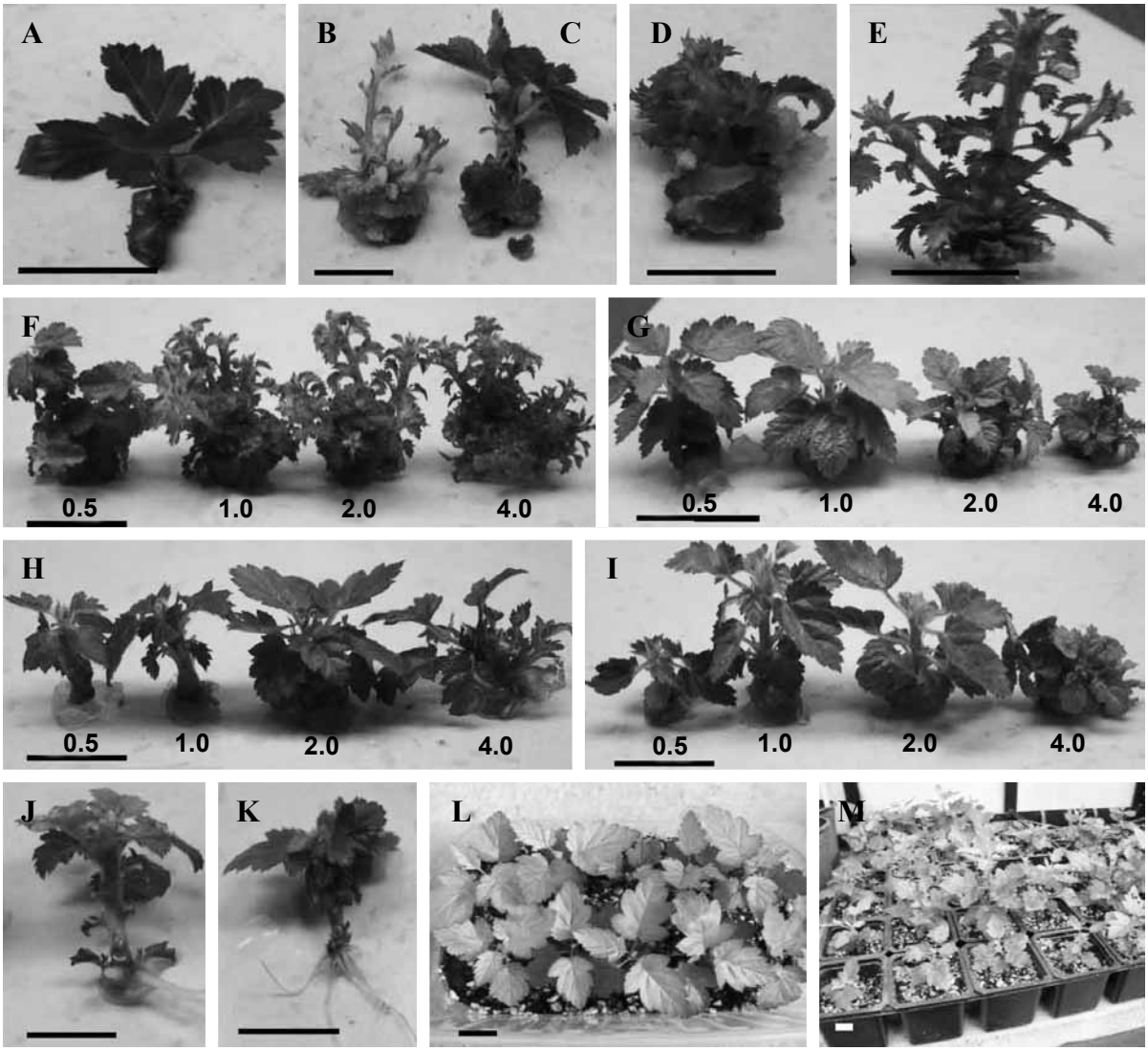

Fig. 1. Typical explant response at the establishment stage on MS medium. A) without plant growth regulators, B) with 1.0 BA + 0.1 IBA (mg l^{-1}), C) with 1.0 zeatin + 0.1 IBA (mg l^{-1}), D) with 1.0 mg l^{-1} TDZ, E) Multiplication on MS medium with 1.0 BA + 0.1 IBA (mg l^{-1}) of explants excised from microshoots produced on MS medium with 1.0 mg l^{-1} TDZ; multiplication on MS medium with F) BA, G) zeatin, H) kinetin, I) 2iP at concentrations shown. Rooted microshoot with J) poor rooting system K) rich rooting system; L) plantlets after 3 week *ex vitro* acclimatization, M) plantlets after 10 week *ex vitro* acclimatization. Size bars = 1 cm.

various reports indicate that TDZ may act through modulation of the endogenous PGRs and others include the modification in cell membranes, energy levels, nutrient uptake, or nutrient assimilation (Murthy et al. 1998, Guo et al. 2011). The number of shoots per explant was higher on variants of the medium with TDZ or BA than on medium with zeatin or without PGRs (control), while longer shoots were formed on variants of the medium with BA or zeatin compared to that with TDZ and the control (Table 2A). Inhibition of shoot elongation by TDZ was also shown for the multiplication stage of ×*M. florentina* (Martini and Papafotiou 2011), for apple (van Nieuwkerk et al. 1986, Fasolo et al. 1989, Pawlicki and Welander 1994) and a number of other woody species (Huetteman and Preece 1993, Lu 1993, Khurana et al. 2005, Parveen and Shahrad 2010). TDZ at low concentrations (0.005-0.05 mg l^{-1}) was used in initial culture and subculture of *Sorbus* as well (Chalupa 1992).

Shoot multiplication

Shoot multiplication is quite successful in ×*M. florentina* (Martini and Papafotiou 2011, Martini et al. 2013). In the present work all explants produced axillary shoots at the subculture on the basal medium independently of the medium used at the establishment stage. The most shoots were produced from explants derived from the TDZ-medium without, falling short in

Table 2. Effect of cytokinin type and concentration on axillary shoot multiplication.

Cytokinin type	Cytokinin concentration (mg l^{-1})	Shooting (%)	Shootnumber per explant	Shoot length (cm)
BA	0.5	100.0 ± 0.0 a (a)	3.1 ± 0.2 c (c)	1.2 ± 0.06 abc (a)
	1.0	100.0 ± 0.0 a (a)	5.3 ± 0.4 b (b)	1.0 ± 0.04 bcd (b)
	2.0	100.0 ± 0.0 a (a)	6.2 ± 0.4 b (ab)	0.9 ± 0.04 def (bc)
	4.0	100.0 ± 0.0 a (a)	7.3 ± 0.4 a (a)	0.8 ± 0.04 efg (c)
zeatin	0.5	100.0 ± 0.0 a (a)	1.3 ± 0.1 ef (b)	1.3 ± 0.07 ab (a)
	1.0	96.7 ± 3.3 ab (a)	1.3 ± 0.1 ef (b)	1.1 ± 0.07 bcd (ab)
	2.0	100.0 ± 0.0 a (a)	2.3 ± 0.2 cde (a)	1.0 ± 0.08 cde (bc)
	4.0	100.0 ± 0.0 a (a)	2.8 ± 0.2 cd (a)	0.7 ± 0.05 efg (c)
kinetin	0.5	16.7 ± 3.3 f (b)	1.0 ± 0.0 f (b)	0.5 ± 0.05 fgh (a)
	1.0	30.0 ± 4.5 ef (b)	1.0 ± 0.0 f (b)	0.5 ± 0.03 gh (a)
	2.0	70.0 ± 4.5 cd (a)	1.1 ± 0.1 f (b)	0.5 ± 0.03 gh (a)
	4.0	66.7 ± 4.2 d (a)	2.0 ± 0.2 def (a)	0.4 ± 0.03 h (a)
2iP	0.5	36.7 ± 3.3 e (c)	1.1 ± 0.1 f (b)	1.0 ± 0.12 bcdef (b)
	1.0	63.3 ± 3.3 d (b)	1.1 ± 0.1 f (b)	1.4 ± 0.09 a (a)
	2.0	83.3 ± 3.3 bc (a)	1.6 ± 0.2 ef (b)	0.9 ± 0.07 def (b)
	4.0	86.7 ± 4.2 ab (a)	2.0 ± 0.2 def (a)	0.6 ± 0.04 gh (c)
Significance of two-way ANOVA:				
$F_{\text{type x concentration}}$		**	**	**

**: Significant at $p < 0.01$; means ± SE within a column followed by the same letter are not significantly different according Tukey-Kramer HSD test at $p < 0.05$ (in parentheses mean comparison per each cytokinin type); $n = 30$.

shoot length (Table 1B, Fig. 1E). This carry over effect of TDZ is quite common and transfer of TDZ-induced shoot clumps in a secondary medium without TDZ or with different balance of PGRs, is a usual practice used to overcome undesirable effects of TDZ (Huetteman and Preece 1993, Khurana et al. 2005, Parveen and Shahrad 2010). TDZ has been previously used in the shoot multiplication stage of ×*M. florentina* (Martini and Papafotiou 2011) stimulating higher shoot multiplication rates than BA; thus a low TDZ concentration (0.1 mg l^{-1}) was suggested in alternative subcultures with basal medium in order to limit the negative effect of TDZ on shoot elongation and subsequent rooting of the microshoots.

Concerning the effect of various cytokinins on axillary shoot multiplication, zeatin, kinetin, and 2iP induced less shoots than BA. Additionally kinetin prevented shoot elongation (Table 2, Fig. 1F,G,H,I). As zeatin gave the most promising results compared to kinetin and 2iP, the experiment was repeated once more, with BA and zeatin only, with similar results (data not shown).

TDZ was not included in this experiment as its effect on proliferation had been previously investigated (Martini and Papafotiou 2011). Shoot multiplication of relative species *Malus* sp. (Hutchinson 1984, Kataeva and Butenko 1987, Lane 1992) and *Sorbus* sp. (Arrillaga et al. 1991, Chalupa 1992, Ördögh et al. 2006) was also stimulated by BA. The most frequently used cytokinin in shoot proliferation of apple is BA (Dobránszki and Texeira da Silva 2010) and in shoot regeneration systems TDZ and BA (Magyar-Tábori et al. 2010). The increase in the concentration of each cytokinin from 0.5 to 4.0 mg l^{-1} led to an increase in shoot number with simultaneous reduction of shoot length (Table 2), which has also been reported for *Malus* sp. (Lane 1992) and *Sorbus* sp. (Chalupa 1992, 2002). Optimum BA concentration for shoot multiplication was between 1.0 and 2.0 mg l^{-1}, similarly to apple (Ma et al. 1990, Lane 1992, Erig et al. 2002, Dobránszki and Texeira da Silva 2010), while in *Sorbus* sp. shoot multiplication was activated by 0.2-2.0 mg l^{-1} BA (Chalupa 1992).

Explants excised from field grown plantlets produced by micropropagation of sprouts (Table 1B) gave less shoots than explants excised from microshoots derived from *in vitro* cultures established from sprouts (Table 2) in agreement with previous report on the effect of explant origin on shoot multiplication (Martini et al. 2013). Cultures established from sprouts produce quite high number of shoots constantly, from the first to the ninth subculture (Martini et al. 2013).

Rooting

The rooting stage showed to be the most critical one in the micropropagation of ×*M. florentina,* as IBA and NAA at various concentrations induced very low rooting and unstable results, and only the combination of IBA with IAA in the "basal technique" mentioned in

materials and methods gave promising results (Savić et al. 2006, Martini 2013, Martini and Papafotiou 2009, 2011, Martini et al. 2013,).

A dark period at the beginning of microshoot rooting has been used successfully in various apple varieties (Welander 1983, Kataeva and Butenko 1987, Bolar et al. 1998), although increased callus was formed (Caboni et al. 1992). In ×*M. florentina* darkness during the root induction period did not affect rooting of both adult and juvenile origin microshoots, while it increased the percentage and the quantity of compact callus produced at the base of the microshoots (Table 3). Similarly in *Quercus euboica* darkness at the beginning of the rooting period did not improve rooting (Kartsonas and Papafotiou 2007), while continuous darkness was essential for rooting microshoots of *Quercus robur* 'Fastigiata' (Pierik et al. 1997).

It has been shown that AC completely inhibited rooting of ×*M. florentina* microshoots derived from an adult plant culture, when added into the root induction medium (Martini and Papafotiou 2009). The presence of AC during the whole root elongation period inhibited rooting of the adult origin microshoots and increased the formation of compact callus at the base of both origin microshoots. The presence of AC during the first week of the root elongation period promoted rooting of microshoots of juvenile origin (Table 3), but not of the adult origin. Partial absorption of auxin by AC (Pan and van Staden 1998) could explain its effect. Alternatively, the establishment of a dark environment at the base of the microshoots could be favorable for root elongation of juvenile origin microshoots. The callus promotion by AC has also been reported for *Pinus pinaster* (Dumas and Monteuuis 1995), *Quercus robur,* and *Q. rubra* (Sanchez et al. 1996). In apple rootstocks, AC presence in the rooting medium reduced rooting percentage but improved the quality of roots (Sharma et al. 2007).

Microshoots derived from a BA-medium rooted at higher percentage than those from zeatin- or 2iP-medium (Table 3), while in the kinetin supplemented medium very few shoots were produced (Table 2) that were not promoted for rooting. Similarly, rooting ability of apple microshoots was affected by cytokinin content of regeneration medium, even after different subculture treatments (Magyar-Tábori et al. 2011).

Microshoots of both adult and juvenile origin produced on a medium with reduced concentrations of BA and IBA compared to that of the basal medium rooted at

Table 3. Factors affecting the rooting of axillary microshoots.

Factor		Callusing (%)	Rooting (%)	Root number	Root length (cm)
Light conditions at root induction					
adult c.	16 h light (*n* = 22)	0.0 ± 0.0 d	31.7 ± 4.2 b	1.3 ± 0.2 a	1.4 ± 0.3 a
	darkness (*n* = 14)	43.3 ± 3.3 b	35.0 ± 5.0 b	1.4 ± 0.2 a	1.8 ± 0.9 a
sprout c.	16 h light (*n* = 139)	32.9 ± 1.4 c	43.2 ± 2.1 a	2.6 ± 0.2 a	1.7 ± 0.2 a
	darkness (*n* = 134)	88.1 ± 1.2 a	47.0 ± 2.8 a	2.2 ± 0.2 a	1.7 ± 0.1 a
AC in root elongation medium					
adult c. (*n* = 72-93)	½MS for 4 w-no AC	41.9 ± 2.0 a	38.7 ± 1.8 b	2.0 ± 0.3 a	3.9 ± 0.4 a
	½MS+AC for 1 w/½MS for 3 w	21.0 ± 2.0 b	27.4 ± 2.2 c	1.5 ± 0.2 a	3.3 ± 0.5 a
	½MS+AC for 4 w	50.0 ± 2.9 a	12.5 ± 2.2 d	1.1 ± 0.1 a	3.5 ± 1.1 a
sprout c. (*n* = 36)	½MS for 4 w-no AC	8.4 ± 3.7 c	30.5 ± 2.8 bc	5.5 ± 1.3 a	2.0 ± 0.3 a
	½MS+AC for 1 w/½MS for 3 w	16.7 ± 0.0 bc	47.2 ± 2.8 a	3.5 ± 0.5 a	1.8 ± 0.2 a
	½MS+AC for 4 w	41.7 ± 3.7 a	33.3 ± 3.2 bc	4.8 ± 1.2 a	2.6 ± 0.4 a
Cytokinin type in multiplication medium					
sprout c. (*n* = 29-36)	BA	19.5 ± 2.8 b	27.8 ± 3.5 a	2.1 ± 0.3 a	2.6 ± 0.6 a
	Zeatin	33.3 ± 3.0 a	16.5 ± 0.4 b	1.4 ± 0.2 a	1.9 ± 0.4 a
	2iP	27.3 ± 3.7 ab	6.7 ± 4.1 b	1.0 ± 0.0 a	4.7 ± 3.7 a
BA+IBA in multiplication medium					
adult c. (*n* = 76-86)	1.0 BA+0.1 IBA	47.6 ± 1.8 b	19.7 ± 1.9 c	2.5 ± 0.4 a	3.7 ± 0.5 ab
	0.5 BA+0.05 IBA	71.0 ± 0.4 a	35.5 ± 2.2 b	2.0 ± 0.3 a	3.3 ± 0.3 b
sprout c. (*n* = 36)	1.0 BA+0.1 IBA	45.7 ± 2.8 b	31.5 ± 2.9 b	1.9 ± 0.4 a	1.6 ± 0.4 b
	0.5 BA+0.05 IBA	5.7 ± 3.5 c	50.0 ± 3.2 a	1.6 ± 0.2 a	5.4 ± 0.7 a

Means ± SE within a column in each rooting factor, followed by the same letter are not significantly different according Tukey-Kramer HSD test at $p < 0.05$.
adult c. = microshoots derived from adult plant cultures;
sprout c. = microshoots derived from sprout cultures.

a higher percentage and formed more roots than those produced on the basal medium (Table 3). This is in accordance with a previous report that ×*M. florentina* microshoots produced on a medium with low TDZ concentration rooted at higher percentage compared to those produced on variant of the medium with high TDZ concentrations (Martini and Papafotiou 2011). Also, microshoots derived from low-cytokinin concentration medium were usually more robust and able to produce more and well developed roots (Martini and Papafotiou 2011). Previous exposure to high cytokinins concentration seemed to inhibit rooting of excised microshoots, which can be attributed to a 'carry over' effect from cytokinins in the shoot proliferation medium (Huetteman and Preece 1993). The presence of cytokinins is essential for induction of cell division at the beginning of root formation (De Klerk et al. 2001), but higher levels of cytokinins inhibit adventitious rooting (Bollmark et al. 1988). If level of endogenous cytokinins is too high, rooting initiation may be inhibited and if it is too low, shoots grow old before becoming autonomous in cytokinin, which happen after the initiation of roots (Bressan et al. 1982).

Microshoots derived from sprout cultures rooted at a higher percentage on an IAA-supplemented medium than on one with IBA or NAA combined or not with IAA (Table 4), in contradiction of what was previously found for microshoots derived from an adult plant culture, which rooted at higher percentages in combinations of 0.5 mg l^{-1} IBA and 2-20 mg l^{-1} IAA than in plain IAA (Martini and Papafotiou 2009). On a medium without PGRs (control) minimum microshoots rooted. The combination of IBA or NAA with IAA gave higher rooting percentages compared to plain IBA or NAA (Table 4). The percentage of callus formation at the base of microshoots, as well as the quantity of callus, were lower on a medium with plain IAA (little callus) compared to variant of the medium with IBA (intermediate callus) or NAA (much callus). There were not differences among variants of the medium in the number and length of roots (Table 4). IAA was preferable to IBA for *in vitro* rooting of *Malus* microshoots too, while NAA gave poor rooting and strongly stimulated the formation of callus (De Klerk et al. 1997, Bommineni et al. 2001). The different effectiveness observed among the three auxins possibly reflects differences in their uptake, transport and metabolism (De Klerk et al. 1997).

Acclimatization

Plantlets from adult and juvenile ×*M. florentina* cultures were easily established *ex vitro* (Fig. 1L,M), similarly to *Sorbus domestica* (Arrillaga et al. 1991), and they did not differ in acclimatization percentage nor in their height (Table 5A). However, plantlets from sprouts formed more nodes than those from adult plants, resulting in a more compact plant shape (Martini et al. 2013). Juvenile clones of apomictic *Malus* sp. also had shorter internodes, along with a greater number of leaves and higher dry weight compared to their mature counterparts (Ur-Rahman et al. 2007), and growth of *Sorbus aucuparia* micropropagated trees was affected by the explant location on adult plants, so that after five years of growth, only the height and diameter of plants from juvenile parts of adult trees were comparable to seedlings growth (Chalupa 2002).

Plantlets that had produced a poor *in vitro* rooting

Table 4. Effect of the auxins, their combination, and concentrations on the rooting of axillary shoots from juvenile cultures.

Auxin concentration (mg l^{-1})	Callusing (%)	Rooting (%)	Root number	Root length (cm)
0.0 (Control)	0.0 ± 0.0 d	7.5 ± 4.8 e	1.5 ± 0.5 a	2.4 ± 1.4 a
0.5 IBA	0.0 ± 0.0 d	22.5 ± 1.4 de	1.0 ± 0.0 a	1.7 ± 0.7 a
0.5 IBA + 4.0 IAA	68.8 ± 5.7 c	46.7 ± 3.3 ab	2.5 ± 0.4 a	1.6 ± 0.4 a
0.5 IBA + 8.0 IAA	72.3 ± 3.6 c	43.8 ± 3.9 abc	2.2 ± 0.5 a	1.6 ± 0.4 a
0.5 NAA	100.0 ± 0.0 a	11.3 ± 6.6 e	2.5 ± 1.5 a	1.4 ± 0.7 a
0.5 NAA + 4.0 IAA	100.0 ± 0.0 a	38.3 ± 4.0 bcd	2.3 ± 0.3 a	2.5 ± 0.3 a
0.5 NAA + 8.0 IAA	100.0 ± 0.0 a	24.3 ± 2.7 de	2.0 ± 0.4 a	2.4 ± 0.3 a
1.0 IBA	77.5 ± 1.4 bc	22.5 ± 1.4 de	1.5 ± 0.5 a	1.2 ± 0.7 a
1.0 IBA + 4.0 IAA	80.5 ± 5.2 bc	38.3 ± 4.0 bcd	1.8 ± 0.3 a	1.6 ± 0.4 a
1.0 IBA + 8.0 IAA	93.8 ± 3.9 ab	40.5 ± 2.2 bcd	3.8 ± 0.9 a	1.3 ± 0.2 a
1.0 NAA	100.0 ± 0.0 a	35.0 ± 6.1 bcd	2.0 ± 0.8 a	1.8 ± 0.3 a
1.0 NAA + 4.0 IAA	100.0 ± 0.0 a	33.8 ± 4.9 bcd	1.7 ± 0.4 a	2.6 ± 0.4 a
1.0 NAA + 8.0 IAA	100.0 ± 0.0 a	37.2 ± 4.1 bcd	1.9 ± 0.4 a	2.3 ± 0.4 a
4.0 IAA	10.0 ± 5.8 d	65.0 ± 5.0 a	2.3 ± 0.4 a	1.4 ± 0.2 a
8.0 IAA	90.0 ± 5.8 ab	65.0 ± 5.0 a	2.6 ± 0.4 a	1.9 ± 0.3 a

Means ± SE within a column followed by the same letter are not significantly different according Tukey-Kramer HSD test at $p < 0.05$; n=17-32.

Table 5. Factors affecting the acclimatization of plantlets.

Acclimatization factor	Acclimatization (%)	Plantlets height (cm)
Physiological state of plantlet		
Adult (*n* = 12)	83.3 ± 9.6 a	29.1 ± 5.4 a
Juvenile (*n* = 24)	83.3 ± 6.3 a	28.5 ± 1.6 a
In vitro root system of plantlets		
Poor (*n* = 48)	27.1 ± 2.1 b	11.3 ± 2.1 b
Rich (*n* = 78)	83.3 ± 3.7 a	16.3 ± 0.9 a
Acclimatization season		
November 2009 (*n* = 40)	65.0 ± 4.7 b	7.1 ± 0.8 b
January 2010 (*n* = 16)	75.0 ± 0.0 ab	11.6 ± 0.9 b
March 2010 (*n* = 59)	84.8 ± 4.2 a	16.6 ± 0.9 a

Means ± SE within a column in each acclimatization factor followed by the same letter are not significantly different according Tukey-Kramer HSD test at $p < 0.05$.
Poor: 1-3 extremely short roots; rich: well developed roots, longer than 0.6 cm).

system acclimatized at a lower percentage and were shorter than plantlets with a well developed rooting system (Fig. 1J,K, Table 5). Vértesy and Balla (1978) had also found significant effect of the root quality (size and number) of woody plant plantlets during their acclimatization.

Plantlets acclimatized at a higher percentage and grew faster when acclimatization started in early spring than in late autumn with survival percentage increasing from November to March (Table 5). Season has also affected acclimatization of apple plantlets with survival percentage steadily increasing from February to June (Vértesy and Balla 1978). This could be attributed to the increase of day length and solar radiation levels in the greenhouse (Average daily solar radiation (kWh m^{-2} day^{-1}): November = 2.9, December = 1.8, January = 1.9, February = 2.8, March = 4.0). Especially radiation, both during *in vitro* development and during acclimatization, constitutes an important factor in the improvement of photosynthetic parameters of plantlets, which ensure their autotrophic growth (Pospíšilová et al. 2007). Rohr et al. (2003) described that enhancing the autotrophic transition of micropropagated plantlets, along with a reduced water stress by adapting culture environment, constitute the two major strategies for successful acclimatization of forest trees.

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