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BRIEF COMMUNICATION

Adventitious rooting performance in micropropagated *Cornus mas*

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Abstract

Axillary buds sampled from a mature 27-year-old *Cornus mas* cv. Macrocarpa were grown *in vitro* on modified woody plant medium (WPM). Adventitious rooting performance of microshoots was assayed on half-strength WPM supplemented with 1-naphthaleneacetic acid (NAA) or indole-3-butyric acid (IBA) under various pH. NAA induced significantly higher rooting frequencies than IBA. The pH of 6.8 inhibited rooting, and differentiated roots were extremely thick and fragile. The highest rooting frequency was recorded on half-strength WPM supplemented with 5.37 μM NAA at the pH value adjusted to 6.2 (73 % of rooted shoots). In the presence of IBA, the formation of adventitious roots was observed only in the basal part of the microshoot dipped into rooting medium. In the case of NAA, however, adventitious roots arose also from the parts of microshoots that were not in contact with medium. The growth of aerial roots was always positively gravitropic. The nuclear microsatellite Cf-G17 gave a monomorphic fingerprinting pattern across the mother shrub and micropropagated plantlets. Acclimatized plants did not show any visually detectable morphological variation and the aerial adventitious root formation was no longer observed.

Additional key words: aerial roots, Cornelian cherry, microsatellite stability, rooting pattern.

Approximately 65 species of genus *Cornus* represent a morphologically diverse group of plants distributed primarily throughout the temperate regions of the northern hemisphere. The majority of species are either small trees or shrubs, have woody rhizomes and characteristic leaf-like appendages subtending the floral disk (Caetano-Anollés *et al*. 1999). A number of species are commercially cultivated, especially cultivars of *C. mas*, *C. florida*, *C. kousa* or *C. nuttalli* for their ornamental characteristics.

Promising results from micropropagation studies were achieved mainly in *C. florida*. Micropropagated plantlets were regenerated either *via* axillary shoot proliferation (Kaveriappa *et al*. 1997) or somatic embryogenesis (Trigiano *et al*. 1989). Attempts were also aimed at micropropagation of other *Cornus* species, including *C. nuttalli* (Edson *et al*. 1994), *C. canadensis* (Pennell 1983) or ornamental cultivars of *C. kousa* (Hadziabdic *et al*. 2004). Adventitious root formation is a crucial step in the vegetative propagation and micropropagation of woody plants. Adventitious roots arise from sites other than their normal sites in the embryo or the primary root. These sites include mainly callus or inner tissues of a severed stem in microcuttings but also nodes on intact plants (Steeves and Sussex 1989).

The aim of this study was to assess *in vitro* adventitious rooting performance under different pH conditions because the pH was shown to be a critical factor affecting elongation and long-term vitality in shoot cultures of *C. mas*.

Twigs with sprouting axillary buds were sampled from a mature 27-year-old mother shrub of *Cornus mas* L. cv. Macrocarpa grown at Arboretum Borová hora of the Technical University in Zvolen, Slovakia. Cultures established *in vitro* were grown on modified woody plant medium (WPM) with changed concentrations of calcium salts and the pH values were adjusted to 6.8 - 7.0 with 0.5 M $Ca(OH)_2$ (Ďurkovič 2008). Media were solidified with 0.6 % agar (*Sigma*, St. Louis, MO, USA), and 2 % sucrose was added. Cultures were maintained at day/night

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Abbreviations: BAP - 6-benzylaminopurine; IBA - indole-3-butyric acid; NAA - 1-naphthaleneacetic acid; PCR - polymerase chain reaction; WPM - woody plant medium.

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temperatures of 25 ± 1/19 ± 1 ºC under a 16-h photoperiod. Light was provided by cool white fluorescent tubes at a photon flux density of 50 μmol m^{-2} s^{-1}. Cultures were regularly sub-cultured every 4 weeks. Shoot multiplication was promoted with 3.11 μM 6-benzylaminopurine (BAP) in combination with 0.27 μM 1-naphthaleneacetic acid (NAA).

Microshoots, more than 1.5 cm in length, were excised from shoot proliferating cultures and transferred to half-strength basal WPM (Lloyd and McCown 1980) supplemented with NAA or indole-3-butyric acid (IBA) at various pH values. The pH was adjusted with 0.5 M $Ca(OH)_2$. Experiments were performed in two replicates with 30 microshoots per each treatment. Rooting frequencies were scored after eight weeks. Shoots in all rooting treatments were maintained at environmental conditions mentioned above. Rooted plantlets were transplanted to small pots with the garden substrate and an addition of powdered lime hydrate. Pots were kept in plastic containers with transparent covers. The regenerants were regularly sprayed with water and grown under high air humidity for three months. Then acclimatized plantlets were transferred to pots with soil and an addition of powdered lime hydrate, and kept under shade in the nursery.

Genomic DNA was isolated using a cetyltrimethylammonium bromide (CTAB), following a procedure modified after Doyle and Doyle (1987). Silica gel-dried leaves (10 - 15 mg) collected from *ex vitro* acclimatized regenerants were used for the extraction. Primers for PCR of a dinucleotide nuclear microsatellite Cf-G17 (Cabe and Liles 2002) were Cf-G17F and Cf-G17R (5'-GAGGGAGCACCTAGTGGA-3' and 5'- GCACCCA CCGCATTTTTAT-3') with the repeat motif $(AC)_n$. PCR amplification was performed in a volume of 0.01 cm^3 of reaction mixture containing 20 ng genomic DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 400 nM of each the forward and reverse primer, and 0.05 U of *Taq* DNA polymerase (*Finnzymes,* Espoo, Finland). Initial denaturation was for 2 min at 94 ºC, followed by 30 cycles of 30 s at 94 ºC, 30 s at 55 ºC, 30 s at 72 ºC and a final 5 min extension step at 72 ºC. Two independent PCR amplifications were performed on a *PTC-200* thermal cycler (*MJ Research*, Waltham, MA, USA) to evaluate the reproducibility. After thermal cycling, 0.005 cm^3 of the amplified product was diluted with 0.005 cm^3 of loading dye. Samples were denaturated at 100 ºC for 2 min before loading 0.005 cm^3 onto 7.4 % pre-electrophoresed vertical denaturing polyacrylamide gel with 6.23 M urea and 0.5× TBE buffer. Electrophoresis was performed on a SEQ-3341 DNA sequencing electrophoresis unit (*Scie-Plas*, Southam, UK) for about 4 h at 39 W. After electrophoresis, microsatellites were resolved by silver staining of the sequencing gel according to the method described in Rajora *et al*. (2000).

Data coming from *in vitro* adventitious rooting experiments were arcsine transformed and subjected to a two-way analysis of variance (*ANOVA*).

Stem microshoots rooted in half-strength WPM supplemented with NAA or IBA under various pH values in the course of 8 weeks. In terms of adventitious rooting frequencies, NAA proved to be a significantly more efficient auxin than IBA in both used concentrations at all pH values (Table 1, $F = 14.32$ at $P = 0.001$). The effect of the pH value 6.8 was shown as inhibitory. On the other hand, no significant differences in rooting frequencies were found between the pH conditions adjusted to 5.8 and 6.2. From the morphological point of view, adventitious roots formed at the pH values 5.8 and 6.2 were characterized by a normal appearance (Fig. 1*A*,*B*), whereas the roots formed at the pH value 6.8 were extremely thick and fragile (Fig. 1*C*). The highest rooting frequency was recorded on half-strength WPM supplemented with 5.37 μM NAA at the pH value adjusted to 6.2 (73 % of rooted shoots, Table 1). In various *Cornus* species, the best adventitious rooting performance was achieved with IBA. In the course of *ex vitro* rooting of *C. nuttalli*, about 53 % of shoots could be rooted by using 4.5 % IBA (Edson *et al*. 1994). In the case of *in vitro* rooting of *C. florida* microshoots, a 52 % frequency was recorded with 2.5 μM IBA and a 46 % frequency with 4.9 μM IBA (Kaveriappa *et al*. 1997). Results with *in vitro* rooting of *C. mas* cv. Macrocarpa are completely different. In term of adventitious rooting percentage, NAA was clearly superior to IBA. This event may be explained by differences on the species level as well as different nutrition requirements for growth. In the case of most cultivars of *C. kousa*, NAA appeared to be a better choice for adventitious root production than IBA (Hadziabdic *et al*. 2004). The positive effect of NAA on root induction was also reported in *Ulmus parvifolia* (Thakur and Karnosky 2007), *Pinus roxburghii* (Kalia

Table 1. Effects of auxins on *in vitro* rooting of *Cornus mas* cv. Macrocarpa shoots after 8 weeks of culture on half-strength WPM with different pH values; means ± SD, $n = 30$ shoots per treatment.

pH	Auxin	Conc. [μM]	Rooting [%]
5.8	control	0	13.3 ± 0.0
	NAA	2.68	53.3 ± 18.9
	NAA	5.37	40.0 ± 9.4
	IBA	2.46	33.3 ± 9.4
	IBA	4.92	23.3 ± 4.7
6.2	control	0	16.7 ± 4.7
	NAA	2.68	66.7 ± 18.9
	NAA	5.37	73.3 ± 18.9
	IBA	2.46	20.0 ± 9.4
	IBA	4.92	16.7 ± 4.7
6.8	control	0	3.3 ± 2.3
	NAA	2.68	43.3 ± 14.4
	NAA	5.37	43.3 ± 14.4
	IBA	2.46	13.3 ± 9.4
	IBA	4.92	16.7 ± 4.7

Fig. 1. Morphology of *in vitro* adventitious roots and different rooting patterns in *Cornus mas* cv. Macrocarpa. Roots formed in the presence of 2.68 µM NAA on half-strength WPM with the pH of 5.8 (*A*), 6.2 (*B*) and 6.8 (*C*), respectively. Roots induced with 2.68 µM NAA (*D* and *E*, on the *left side*) were initiated above the surface of rooting medium, whereas roots induced with 2.46 µM IBA differentiated inside the rooting medium (*D* and *E*, on the *right side*). *F* - Close-up view of the first axillary shoot axil from which the aerial adventitious root outgrows after exposure to NAA. *G* - Aerial roots outgrowing from microshoot nodes towards opposite sides after exposure to NAA. *H* - Aerial roots oriented spatially to the equal side after exposure to NAA.

et al. 2007) or *Maytenus canariensis* (Gutiérrez-Nicolás *et al*. 2008).

Besides significantly higher rooting frequencies, NAA frequently induced aerial adventitious roots. In general, the site of *in vitro* adventitious root formation in microshoots is associated with the basal part of the shoot that is dipped into rooting medium. This common pattern was found in microshoots that rooted mainly in the presence of IBA. With regard to NAA, the additional patterns were observed. In most cases and regardless of concentration, adventitious roots arose from callus or inner tissues of the part of microshoot that was at the interface between rooting medium and air inside a test tube. These roots differentiated above the surface of rooting medium and elongated into the medium. Comparisons of *in vitro* rooting patterns induced by NAA

and IBA (Fig. 1*D*,*E*) showed several unusual rooting patterns induced by NAA. The common feature in all these examples is that the site of adventitious root formation was associated with aerial parts of the shoot not in contact with medium (Fig. 1*F*). Unusual examples were also observed from nodes along the shoot. The aerial roots were oriented spatially either to opposite sides (Fig. 1*G*) or to the equal side (Fig. 1*H*). The growth of all main aerial roots was always positively gravitropic. In some cases, root initiation starts from other than stem tissues. For instance in mature petioles of *Hedera helix* cultured *in vitro*, roots were initiated from the callus formed from dividing cortical cells, but in juvenile petioles of the same species, root initiation started in epithelial cells associated with ducts adjacent to the vascular bundles (Geneve *et al*. 1988). In *Liquidambar formosana* microshoots cultured *in vitro*, aerial adventitious rhizogenesis was observed from the aerial parts not in contact with rooting medium, including petiole axils or vascular parenchyma cells surrounding the main vein on the abaxial side of leaf (Ďurkovič *et al*. 2005). Similar aerial rooting patterns were also found in *C. mas* cv. Macrocarpa. In this case, aerial adventitious root formation was associated with the axillary shoot axils or with nodes bearing axillary buds. In all these examples, unusual rooting was initiated only after exogenous addition of NAA into rooting medium.

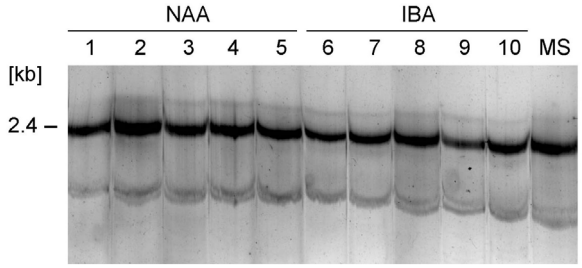

Fig. 2. Monomorphic amplification pattern obtained for plantlets rooted in the presence of NAA (*lanes* 1 - 5), IBA (*lanes* 6 - 10) and the mother shrub (MS) of *Cornus mas* cv. Macrocarpa by using Cf-G17 microsatellite primer.

Due to different patterns of *in vitro* adventitious rooting triggered by widely used auxins NAA and IBA, we wanted to compare the microsatellite stability in rooted plantlets acclimatized to an *ex vitro* environment. The microsatellite primers Cf-G17 generated the scorable band class with the molecular size of 2.4 kbp. These bands gave rise to a monomorphic pattern across plants rooted with NAA, IBA, and the mother shrub (Fig. 2).

Acclimatized plantlets were potted in a soil mixture with an addition of powdered lime hydrate and kept under shade. Eighty percent of plantlets survived the transfer. All acclimatized plantlets did not show any detectable morphological variation and the aerial adventitious root formation was no longer observed. Taken together with the observed morphological homogeneity of acclimatized plantlets without the visible variation, there is a strong suggestion that mechanisms of NAA action are involved in both anaerobic and aerial root formation in *C. mas* cv. Macrocarpa.

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