



DOES SHOOT ANATOMICAL HETEROGENEITY INFLUENCE *EX VITRO* ADVENTITIOUS ROOT FORMATION IN RHODODENDRON MICROCUTTINGS?

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Abstract

Rhododendrons (*Rhododendron* L., Ericaceae) are among the most popular ornamental plant species in Latvia. Mostly they are propagated by rooting of macrocuttings or microcuttings *ex vitro*. Sometimes the result of *ex vitro* adventitious rooting is unsuccessful even when using morphologically uniform microcuttings and identical propagation conditions. Thus the aim of this study was to determine how the successive adventitious rooting phases are achieved and to check whether there are any initial anatomical differences between microshoots that could affect rooting. Elepidote rhododendron 'Nova Zembla' adventitious shoots were multiplied *in vitro* and rooted *ex vitro*. Anatomical observations were done during the experiment and measurements of vascular cylinder : shoot diameter and phloem : xylem width were done. The initial plant material was very heterogeneous – different stem developmental stages were observed. Some microshoots had a continuous vascular ring while others had separate vascular bundles. First rooting related changes were visible on day 4, when groups of dividing meristematic cells were observed in cambial region. First root primordia were found on day 12, and first roots were observed on day 16. During the whole experiment shoots were morphologically uniform but both the vascular cylinder : shoot ratio and phloem : xylem ratio differed. Microshoots that formed meristemooids had larger phloem : xylem ratio. Those microshoots which did not undergo any rooting-related changes had smaller phloem : xylem ratio. Thus we suggest that the development of microshoot vascular system might play significant role in adventitious rooting processes.

Key words: anatomy, *ex vitro*, microshoot heterogeneity, propagation, rhizogenesis, *Rhododendron*

INTRODUCTION

Clonal micropropagation of many important ornamental and crop plants can be very useful when there is a limitation to plant material or when there is a need to eliminate specific virus diseases (George et al. 2008, Hartmann et al. 2011). Cuttings produced in tissue culture (microcuttings) may be rooted *in vitro* or *ex vitro* with or without auxin treatment (George et al. 2008). Often the limiting step of the micropropagation is rooting of microcuttings (San-José et al. 1992). Both *in vitro* and *ex vitro* rooting conditions have their advantages. *In vitro* root formation is preferred because it takes part under controlled conditions like auxin and other compound administration, avoidance of microbial degradation of applied compounds, addition of inorganic nutrients and carbohydrates etc. (De Klerk et al. 1999). *Ex vitro* rooted plants have roots with better developed vascular system (San-José et al. 1992)

and better acclimatized to new environment during the rooting process (Hatzilazarou et al. 2006).

Most studies on adventitious root formation in microcuttings are carried out *in vitro* (San-José et al. 2012) and information on *ex vitro* rooting of microcuttings is very scarce. Hitherto the main anatomical (De Klerk 2002) and physiological (Gaspar et al. 1994) phases have been recognized. Adventitious root formation both *in vitro* and *ex vitro* follows series of successive stages which can be defined anatomically as dedifferentiation, induction, and differentiation (De Klerk 2002). Physiologically these phases are defined as induction, initiation and expression (Gaspar et al. 1994).

Rhododendrons (*Rhododendron* L., Ericaceae), one of the most popular ornamental woody plants in Latvia, can be propagated either by rooting of cuttings or *in vitro*. Little results concerning rhododendron microshoot anatomy and *ex vitro* rooting have been published. Last year experience shows that different *ex vitro*

rooting percentages can be achieved despite constant *in vitro* multiplication conditions and morphologically uniform microcuttings (unpublished results). The aim of this study was to find out whether these differences are related to anatomical peculiarities.

MATERIALS AND METHODS

The experiment was carried out in Plant Tissue Culture Laboratory, University of Latvia from March till December 2012 using *in vitro* propagated elepidote rhododendron ‘Nova Zembla’ from the collection of the laboratory.

Rhododendron shoots were cut in nodal segments (~1 cm in length) with two leaves and placed on modified Anderson medium (Anderson 1984) with sucrose 20 g l⁻¹, inositol 0.1 g l⁻¹, adenine 0.04 g l⁻¹, casein hydrolysate 1 g l⁻¹, thiamine 0.4 mg l⁻¹, pyridoxine 0.1 mg l⁻¹, nicotinic acid 0.1 g l⁻¹, agar 7.9 g l⁻¹, N⁶-(2-Isopentenyl)adenine (2iP) 3 mg l⁻¹, pH 5.5. Cultures – 30 nodal segments per magenta – were incubated at 23 ± 2°C under cool white fluorescent light (50 mmol m⁻² s⁻¹) with a 16-h photoperiod for three months.

After 3 months 2 cm large adventitious shoot apical fragments were taken for *ex vitro* rooting in peat (70 % milled peat, 30% peat moss, with 0-5 mm structure, pH/KCl 4.5 ± 0.3). Microshoots were inserted in plastic beds covered with a polyethylene tent and maintained in a growth chamber under a 16 h photoperiod (23°C day, 20°C night).

On day 0 and every second day, for 18 days, 3-4 cuttings were fixed in FAA solution (37% formaldehyde : 95% ethanol : acetic acid : distilled water (10 : 50 : 5 : 35)) for a histological studies (Ruzin 1999). After fixation, tissues were dehydrated in an ethanol-tert-butyl alcohol series, and embedded in Histowax (HistoLab) (Ruzin 1999). Serial 15 µm cross sections at the basal end (0-0.5 cm) of microshoots were prepared using a

rotary microtome (Leica RM2145), deparaffinized in a xylol-ethanol series, stained with astra blue - safranin (Braune et al. 1999), dehydrated in an ethanol-xylol series, and mounted on glass slides in Canada balsam. Sections were examined and photographed using a Leica DM5500B light microscope equipped with a digital camera Leica DFC490.

Stem anatomical measurements - vascular cylinder : shoot diameter ratio (V : S) and phloem : xylem width ratio (P : X) – were performed in Image Pro 6. Four microcuttings were measured at day 0, four microcuttings with root meristemoids, two microcuttings with roots and four unrooted microcuttings were measured during the experiment. Three measurements of each parameter were done per microcutting. Obtained data were analyzed in IBM SPSS Statistics 20. Significant differences between groups were assessed using Tukey test.

RESULTS

The observations revealed that the initial plant material was very heterogeneous. Stems of microcuttings consisted of epidermis, cortex, phloem, cambium, xylem, and pith (Fig. 1A,B, Fig. 2). At the beginning of the experiment the collenchyma layer and the endodermis were not formed. The development of sclerenchyma was not observed (Fig. 1B). Epidermis was one cell layer thick, with multicellular trichomes (Fig. 1A). Cortex consisted of large, vacuolated parenchyma cells. Vascular cylinder consisted of phloem, cambium, and xylem (Fig. 1B, Fig. 2). In some plants the vascular cylinder was not formed in continuous ring, and separate vascular bundles were visible (Fig. 1A). There was a discontinuous (Fig. 1B) or continuous (Fig. 2) cambial zone with 1-2 layers of cells between the phloem and xylem. The phloem consisted of sieve tube elements and companion cells (Fig. 1B). The xylem consisted of

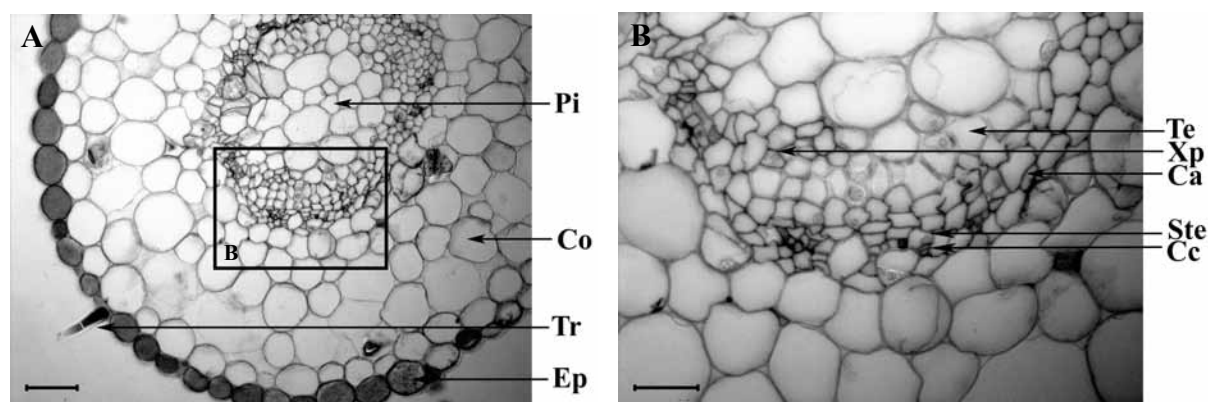


Fig. 1. A) Cross section of elepidote rhododendron ‘Nova Zembla’ microcutting with differentiating vascular tissues on day 0. P - pith, Co - cortex, Tr - trichome, Ep - epidermis. Scale bar: 30 µm, B) Differentiating vascular tissue of elepidote rhododendron ‘Nova Zembla’ microcutting on day 0. Te - tracheary element, Xp - xylem parenchyma, Ca - Cambium, Cc - companion cell, Ste - sieve tube element. Scale bar: 10 µm.

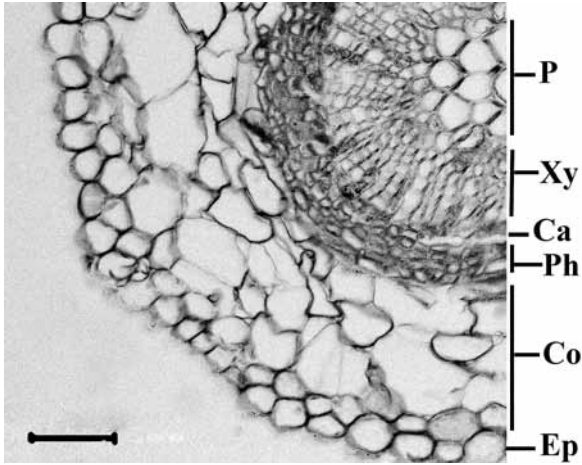


Fig. 2. Cross section of elepidote rhododendron 'Nova Zembla' microcutting with developed vascular tissues on day 0. P - pith, Xy - xylem, Ca - cambium, Ph - phloem, Co - cortex, Ep - epidermis. Scale bar: 50 μ m.

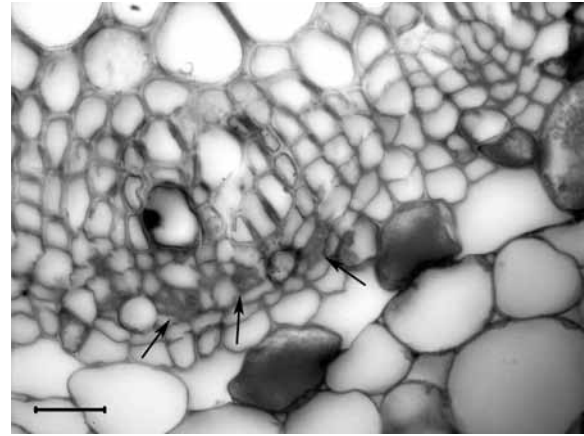


Fig. 3. Clusters of cambial derivatives (arrows) in phloem region of elepidote rhododendron 'Nova Zembla' microcutting on day 4. Scale bar: 10 μ m.

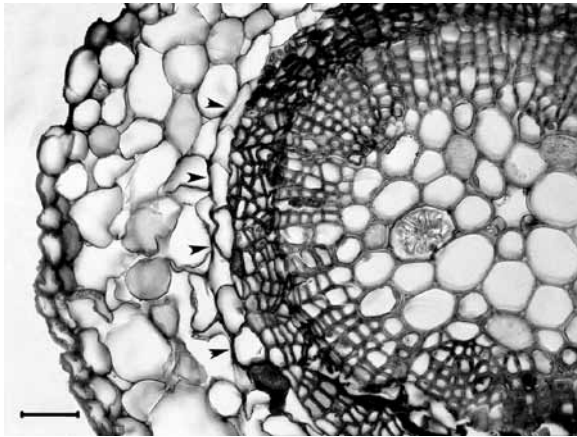


Fig. 4. Formation of endodermis (arrowheads) in elepidote rhododendron 'Nova Zembla' microcutting on day 6. Scale bar: 50 μ m.

groups of lightly lignified tracheary elements, parenchyma cells were present as well. The pith was located in the middle of the stem.

On day 4 round groups of densely stained meristematic cells in phloem region were found (Fig. 3).

On day 6 an endodermis-like layer was observed for the first time. Cells in phloem region formed stacks, some of them were densely stained, nuclei were also visible (Fig. 4).

Further no obvious anatomical changes were observed until day 12, when root primordia were observed (Fig. 5A,B) and it seemed like all of them were formed in cambial region. They were elliptic structures consisting of enlarged cells (Fig. 5A), which formed root cap, cortex initial cells, pericycle, and vascular tissues (Fig. 5B). All these meristematic cells were densely stained and contained large nuclei.

On day 16 roots had already emerged outside and some of them had already started to form lateral roots

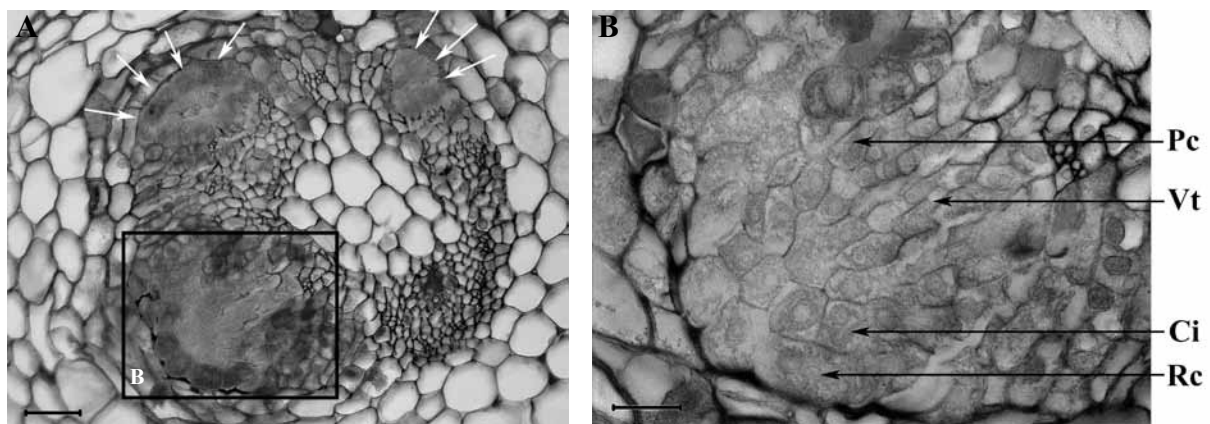


Fig. 5. A) Primordia (arrows) in elepidote rhododendron 'Nova Zembla' microcutting on day 12. Scale bar: 30 μ m, B) Primordium in elepidote rhododendron 'Nova Zembla' microcutting on day 12. Rc - root cap, Ci - cortex initial cells, Pc - pericycle, Vt - vascular tissues. Scale bar: 10 μ m.

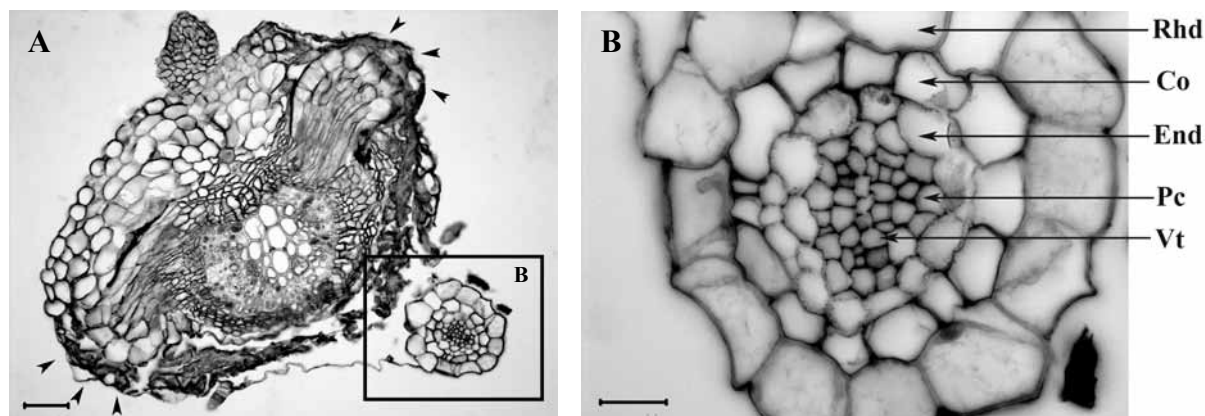


Fig. 6. A) Emerging primordia (arrowheads) and an adventitious root (6B) in elepidote rhododendron ‘Nova Zembla’ microcutting on day 16. Scale bar: 50 µm, B) An adventitious root of elepidote rhododendron ‘Nova Zembla’ microcutting on day 16. Rhd - rhizodermis, Co - cortex, End - endodermis, Pc - pericycle, Vt - vascular tissues. Scale bar: 10 µm.

(Fig. 6A). Newly formed roots consisted of differentiating vascular cylinder surrounded by four cell layers, which were defined as pericycle, endodermis, cortex, and rhizodermis (Fig. 6B).

Anatomical measurements done during the experiment allowed calculation of vascular cylinder : shoot diameter (V : S) ratio and phloem : xylem (P : X) width ratio (Table 1). At the beginning of the experiment V : S ratio was very heterogeneous (0.35-0.51; results not shown) P : X ratio differed even more (0.46-1.08; results not shown), which means that in some plants there was twice as more xylem as phloem, and in some plants the ratio was almost 1 : 1. Meristemoids and roots were observed in microcuttings with higher V : S and P : X ratios. During the whole experiment microcuttings with relatively lower ratios of both V : S and P : X did not form any adventitious rooting related structures. There was no correlation between V : S ratio and P : X ratio in any of the rooting stages (Table 1). The Tukey test showed significant difference between P : X ratios of rooting and non-rooting microcuttings.

Table 1. Vascular cylinder : shoot ratios and phloem : xylem ratios during different *ex vitro* adventitious rooting stages of elepidote rhododendron ‘Nova Zembla’ microcuttings.

Stage	V/S ± SE	P/X ± SE
Beginning	0.418 ± 0.015 b	0.662 ± 0.062 a
Meristemoid	0.534 ± 0.018 a	0.789 ± 0.025 a
Root	0.426 ± 0.023 b	0.730 ± 0.040 a
Non-rooted	0.473 ± 0.016 ab	0.435 ± 0.021 b

Legend: V/S – vascular cylinder : shoot ratio, P/X – phloem : xylem ratio. Means ± standard error (SE) followed different letters are significantly different at $p \leq 0.05$ probability level.

DISCUSSION

Rhododendron stems usually consist of structures typical to woody plants, i.e., in the middle of the stem there is pith, a continuous ring of xylem and phloem with the cambium between. Vascular tissues are surrounded by continuous ring of sclerenchyma and endodermis. The outer layers of stem are cortex, collenchyma and epidermis (Yeremin and Boiko 1998, Kondratovičs and Megre 1999, Megre et al. 2011). *In vitro* microshoot anatomy is similar to stem anatomy of *in vivo* rhododendron plants, except for lack of collenchyma, endodermis, and sclerenchyma (Fig. 1A,B, Fig. 2). It is known that collenchyma tissues are especially suited to provide support to young plant organs (Leroux 2012) but in young microshoots of elepidote rhododendron collenchyma was not observed similarly to the observations in *Cedrela odorata* (Millán-Orozco et al. 2011) and *Alnus glutinosa* (San-José et al. 2012). Donnelly et al. (1985) had the same observations and they suggested that it might be related to the lack of mechanical stress in culture conditions. During the experiment the collenchyma layer was not formed at all, contrary to the endodermis, which was observed on day 6. Often the endodermis of woody plants has been described as a starch sheath (Fahn 1990). Probably because of the high sugar content in the multiplication medium *in vitro* or because of the limited photosynthetic rate in *ex vitro* conditions the endodermis was not present in elepidote rhododendron microcuttings. The development of sclerenchyma layer *in vitro* is very variable between different woody plant species, for example it was abundant in *Castanea sativa* (Ballester et al. 1999), *Alnus glutinosa* (San-José et al. 2012), and *Cedrela odorata* (Millán-Orozco et al. 2011). Similarly as in our experiment lack of sclerenchyma has been reported in *Malus domestica* Borkh. ‘Gala’

(Harbage et al. 1993) where formation of the secondary phloem was hardly underway. Zhou et al. (1992) reported a few sclerenchyma fibres in some regions of *Malus domestica* M26 microcuttings. Donnelly et al. (1985) suggested that scarcity of sclerenchyma fibers and secondary wall development might be related to relatively high humidity in culture environment.

The main adventitious root formation related processes occur within days or even hours after setting microcuttings into rooting medium. These processes have been divided in three phases (De Klerk 2002). The first phase is the dedifferentiation during which the reactivation of cell cycle happens in dedifferentiated and differentiating cells (Altamura 1996). This phase lasts approximately 24 h. As a result these cells become competent to respond to rhizogenic signals (De Klerk 2002). During the second phase, which is the induction phase, the first cell divisions occur (De Klerk 2002). In our experiment the first cell divisions in the cambial region were observed on day 4 (Fig. 3). As a result groups of meristematic cells were formed. It is considered to be the key stage in adventitious root formation (Altamura 1996). Usually it takes 3-4 days for the first meristemoids to be formed (De Klerk 2002, Millán-Orozco et al. 2011). The first root primordia were visible only on day 12 (Fig. 5A,B). The presence of different root primordia developmental stages on day 12 indicates that previously meristemoids have not formed simultaneously. While the meristemoids were growing and differentiating the main root anatomical structures were developed as described by Ermel et al. (2000). Altamura (1996) stated that growing primordia inhibit other meristemoids from developing into primordia. Contrary to these trends, similarly to Iliev et al. (2001) we observed primordia of different developmental stages in one explant. First roots were observed on day 16 (Fig. 6A,B). Four layers of larger cells surrounding the central part of smaller cells were visible in cross sections of newly formed roots (Fig. 6B). The outer layers probably could be pericycle, endodermis, cortex, and rhizodermis and in the middle there were differentiating vascular tissues, it was not possible to define their type with light microscopy.

Anatomical measurements done during the experiment showed that the microcutting heterogeneity cannot be seen morphologically, i.e., thicker cuttings could have less developed vascular system and vice versa (Table 1). Correlation between stem diameters and vascular tissue width was not found. Adventitious rooting related structures did not form in microcuttings with weakly developed phloem (in microcuttings with low P : X ratio, respectively) and microcuttings with developed phloem did form adventitious root meristemoids and primordia. This could be explained by disturbed endogenous hormonal balance after removing the microshoots of the *in vitro* medium. In fact, Reverberi et

al. (2001) in their studies reported a tendency to xylem formation in *Juglans regia* L. microcuttings when there is disbalance in auxin metabolism. In their experiment an exogenous indole-3-acetic acid was used, which increased the endogenous IAA level and led to xylem differentiation and inhibited the phloem differentiation as well as the adventitious root formation.

In conclusion we suggest that initial microshoot heterogeneity, and especially the development of vascular tissues play significant role in the *ex vitro* adventitious root formation processes and timing and it should be considered when discussing the results of different rooting experiments. In recent studies initial microshoot anatomical heterogeneity has not been considered as influential factor, in fact, it has not been discussed at all, but in some cases the loss of plant material might be related to these anatomical differences and weakly developed vascular system in explants. We suggest that microshoot anatomical differences should be considered in future research.

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