



# CAN CHANGES IN STARCH CONTENT AND PEROXIDASE ACTIVITY BE USED AS ROOTING PHASE MARKERS FOR RHODODENDRON LEAF BUD CUTTINGS?

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Received November 18, 2010; revision accepted April 5, 2011

We examined whether peroxidase activity in cutting bases and leaves and starch content in cutting bases can be used as rooting phase markers in the elepidote rhododendron cv. 'Babites Baltais' (*Rhododendron* L.). Changes in peroxidase activity in cutting leaves and bases, as well as starch content in cutting bases, were determined in relation to anatomical stages of rhizogenesis in leaf bud cuttings treated with 1% indole-3-butyric acid (IBA+) or without IBA (IBA-). The pattern of change of peroxidase activity was similar in cutting bases and leaves of IBA- leaf bud cuttings. Three phases of adventitious root formation were identified: induction, initiation and expression. During the induction phase peroxidase activity decreased, but no anatomical changes were observed in the cuttings. Peroxidase activity increased in the initiation phase when adventitious root initials were formed. Peroxidase activity decreased during the expression phase when adventitious root primordia developed. The starch content of IBA- leaf bud cuttings decreased during the first few days and then gradually rose to maximum, followed by a sharp reduction and another increase at the end of the experiment. The changes of starch content did not coincide with rooting phases as peroxidase activity did, and cannot be used as a rooting phase marker in rhododendrons. Adventitious root formation did not occur in IBA+ leaf bud cuttings, so distinct rooting phases could not be observed. There was a significant correlation between peroxidase activity in cutting bases and leaves of IBA- leaf bud cuttings. Peroxidase activity in leaves of rhododendron leaf bud cuttings are potentially useful as a marker for rooting phases, but that requires further anatomical and physiological study of rooting in leaf bud cuttings.

**Key words:** Peroxidase, starch, adventitious root, leaf bud cuttings, rhododendron.

## INTRODUCTION

Rhododendron can be propagated by seed or vegetative methods. Vegetative propagation is an important commercial method of producing large quantities of genetically uniform plant material. Conventionally, elepidote (evergreen) rhododendrons are propagated by cuttings, grafting and tissue culture. One method of rhododendron propagation is by leaf bud cuttings. This method is particularly useful when propagation material is scarce, because it will produce at least double the number of new plants from a given amount of stock material than from stem cuttings (Hartmann et al., 2002). Adventitious root formation in some plant species initiates without any special treatment, while other species require supply of different growth regulators, usually auxins (Hartmann et al., 2002). It has been suggested that double wounding of rhododen-

dron stem cuttings greatly increases the total percentage of well-rooted large-flowered evergreen rhododendron (Dirr and Heuser, 1987), and that the best rooting stimulant for evergreen rhododendrons is IBA (indole-3-butyric acid) (Sanders, 1978; Goreau, 1980; Nawrocka-Grześkowiak, 2004) at concentrations ranging from 0.8% to 2% depending on the species and cultivar (Hartmann et al., 2002).

One of the main achievements in the study of adventitious rooting has been the recognition of interdependent phases (induction, initiation and expression) (Gaspar et al., 1992). These phases have been identified through variation of some biochemical markers. At present, peroxidase is asserted to be the best rooting phase marker for rooting of cuttings (Gaspar et al., 1992; Arena et al., 2003; Metaxas et al., 2004; Syros et al., 2004; Naija et al., 2008;). The induction phase of rooting, before any visible morphological and histological events, is

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characterized by a decline in peroxidase activity, and the minimum of peroxidase activity indicates termination of this phase. A period of high peroxidase activity and cell dedifferentiation followed by cell division are characteristic of the initiation phase. In the expression phase there is a gradual drop in peroxidase activity accompanying the organization and growth of root primordia (Gaspar et al., 1994). Such changes of peroxidase activity have been reported also in *Rhododendron catawbiense* Michx. cv. *album* cuttings in vitro (Aghmir et al., 1991). We assumed that changes in peroxidase activity can also be used as a biochemical marker of rooting in leaf bud cuttings of evergreen rhododendron. Investigations of typical peroxidase variation during rooting have focused mostly on cutting bases, but in cutting leaves it has been assessed in only a few studies (Fekete et al., 2002; Ludwig-Müller, 2003). In *Helichrysum stoechas* (L.) Moench cuttings, trends of peroxidase activity during rooting were observed to be similar in leaves and cutting bases (Fekete et al., 2002), so we might expect these peroxidase activity trends during the adventitious root formation to be similar in bases and leaves of elepidote rhododendron cuttings.

Cell division and cell enlargement during adventitious root formation require high inputs of energy and carbon. A major source of carbon is sucrose, which is formed in photosynthetically active tissues and translocated towards the stem base either to be used after cleavage into hexoses as a direct carbon source or to be converted to storage compounds such as starch (Ahkami et al., 2009). Although there is much correlative data showing the important role of carbohydrates in adventitious root formation, the precise functions of carbohydrates in different developmental phases are still unknown (Klopotek et al., 2010). According to Apine and Kondratovics (2005), the starch content in cuttings of deciduous azalea was better related to the rooting process than was the content of total soluble sugars, suggesting that the concentration of starch in cuttings might be used as an indicator of the rooting process.

In this study we examined whether peroxidase activity in cutting bases and leaves and starch content in cutting bases can be used as markers of rooting phases in elepidote rhododendron leaf bud cuttings. Trends in peroxidase activity (cutting leaves, bases) and starch content (cutting bases) were determined in relation to anatomical changes during rooting of rhododendron leaf bud cuttings.

## MATERIALS AND METHODS

The investigation was carried out from October to December 2007 and used the elepidote cultivar 'Babites Baltais' ('Cunningham's White' × 'Elisabeth')

of *Rhododendron* L. bred by Kondratovics (Leslie, 2006). A leaf bud cutting consists of a leaf blade, petiole and a piece of stem ~2.5 cm long with the attached axillary bud. The plant material was obtained from the Babite Experimental Nursery of *Rhododendron* Breeding, University of Latvia. Basal ends (0.5–0.7 cm) of leaf bud cuttings were dipped in 1% indole-3-butyric acid (IBA) on talc powder (IBA+ cuttings), or only talc powder (IBA– cuttings), and inserted in plastic beds. Soil consisted of a mixture of peat moss and pine needles. Leaf bud cuttings were covered with a polyethylene tent and maintained in a growth chamber with a 16 h photoperiod (23°C day, 20°C night) under fluorescent lamps (Osram L36 W/77 Fluora; PPFD 80  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ).

Three leaf bud cuttings were collected on days 0, 3, 6, 9, 12, 15, 18 and 21 of the experiment. Leaf bud cuttings were fixed in FAA solution (37% formaldehyde, glacial acetic acid, 95% ethanol, distilled water; 10:5:50:35, v/v/v/v). After fixation, tissues were dehydrated in an ethanol-tert-butyl alcohol series and embedded in Histowax (Ruzin, 1999). Serial 25  $\mu\text{m}$  cross sections were prepared using a rotary microtome (Leica RM2145), deparaffinized in a xylol-ethanol series, stained with astra blue-safranin, dehydrated in an ethanol-xylol series and mounted on glass slides in Canada balsam (Braune et al., 1999). Sections were examined and photographed with a Leica DM5500B light microscope equipped with a Leica DFC490 digital camera.

For peroxidase measurement, 12 cuttings were taken (4 replicates of 3 cuttings each). Cutting bases (~2 cm = 0.250 g) and cutting leaves (0.5 g) were frozen in liquid nitrogen and ground to a fine powder with mortar and pestle. Enzymes were extracted with 25 mmol l<sup>-1</sup> HEPES /KOH buffer (pH 7.2) containing 1 mmol l<sup>-1</sup> EDTA, 3% (w/v) PVPP (polyvinylpyrrolidone) and 0.8% (v/v) Triton X-100 (polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether) for 15 min at 4°C. The homogenate was centrifuged at 15,000 g for 20 min. The supernatant was used for assay. Peroxidase activity was measured spectrophotometrically at 470 nm in a reaction mixture containing 2 ml 50 mmol l<sup>-1</sup> sodium phosphate buffer (pH 7.0) with 10 mmol l<sup>-1</sup> guaiacol, 0.5 ml 0.03 mol l<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> and 0.01 ml enzymatic extract. Reaction mixture without H<sub>2</sub>O<sub>2</sub> was used as the reference (Andersone and Ievinsh, 2002).

For starch analysis, 30 bases of leaf bud cuttings were taken (3 replicates of 0.5 g dry mass each). Dry plant material was ground with 10 ml 80% (w/v) Ca(NO<sub>3</sub>)<sub>2</sub> solution and boiled for 3 to 5 min to pass starch into the colloidal solution. Starch content was determined by the Berthram method of bichromate-sodium thiosulfate titration (Strong and Koch, 1974) and is expressed as percentage of dry mass.

To test the significance of the relationship between the levels of peroxidase in cutting bases and leaves, Pearson correlation coefficients ( $p < 0.05$ ) were determined using SPSS 17.0 for Windows.

## Results

The stem anatomy of leaf bud cuttings was typical of woody stems; vascular tissue formed a continuous ring of phloem and xylem (Fig. 1a). A continuous ring of sclerenchyma fibers was present between the cortex and phloem (Fig. 1a). The cells of phloem rays became isodiametric and started to divide, producing the root initial. Root initial formation from phloem ray parenchyma was first observed on day 9 in IBA- cuttings (Fig. 1b). Polarization of division in the root initials gave rise to a typical dome-shaped root primordium which pushed through the ring of sclerenchyma fibers to reach the cortex tissue (Fig. 1c). However, adventitious root emergence on the surface of the cuttings was not observed until day 21. We did not note any adventitious root initial formation in IBA+ leaf bud cuttings.

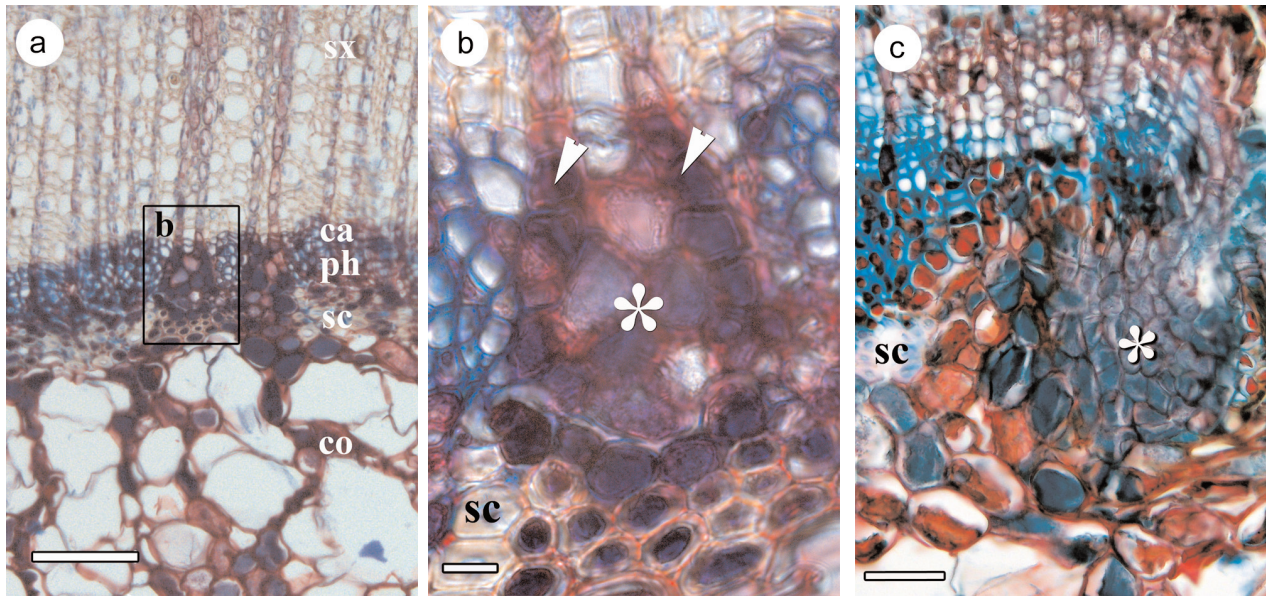
The pattern of peroxidase activity change during adventitious root formation (from days 0 to 21) in the cutting bases and leaves differed between the IBA- and IBA+ leaf bud cuttings (Fig. 2). There was a significant ( $r^2 = 0.714$ ,  $p < 0.05$ ) correlation of peroxidase activity between bases and leaves of IBA- leaf bud cuttings. In both bases and leaves from IBA- leaf bud cuttings, peroxidase activity fell to minimum on day 6 and rose to maximum on day 12, with a subsequent decrease up to day 15, followed by an increase (Fig. 2a).

In IBA+ leaf bud cuttings the first peak of peroxidase activity in cutting bases appeared on day 3 (Fig. 2b). Then the level began to decrease from days 3 to 12, with a second peak on day 15 (Fig. 2b). There was no clear change in peroxidase activity in leaves of IBA+ leaf bud cuttings and no significant correlation in peroxidase activity between bases and leaves of IBA+ leaf bud cuttings (Fig. 2b). Initial starch content was high in the cutting bases in both IBA- and IBA+ leaf bud cuttings. Starch content in IBA- leaf bud cuttings decreased from days 0 to 3 and then gradually rose to maximum on day 15, followed by a decline up to day 18 and then an increase to day 21 (Fig. 3). Interestingly, on day 9 the appearance of the first root initials in IBA- leaf bud cuttings did not influence the increase of starch content. In IBA+ leaf bud cuttings the starch content began to decrease from days 0 to 9, with a peak on day 12 and later an increase from day 15 (Fig. 3).

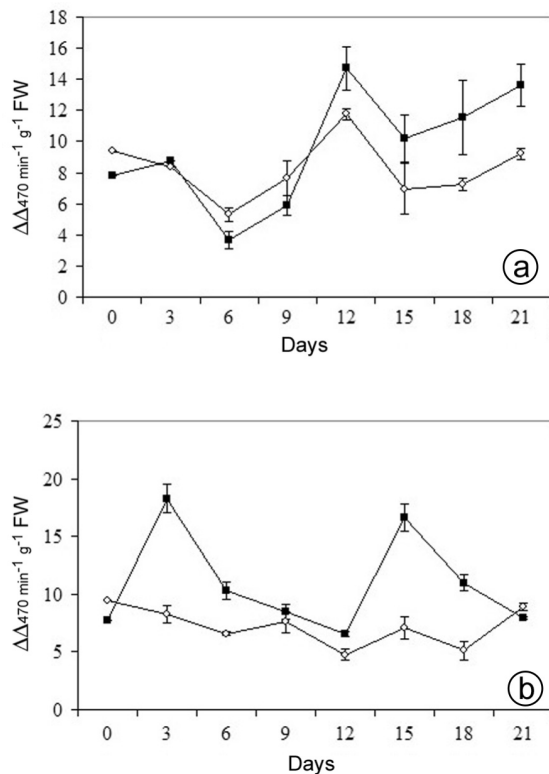
## DISCUSSION

Many authors have examined the relationship between different aspects of rooting and changes in peroxidase and isoperoxidase activity. Peroxidase activity has been used to define rooting phases (induction, initiation and expression) (Gaspar et al., 1994) in several species: *Elaeis guineensis* (Rival et al., 1997), *Psoralea corylifolia* (Rout et al., 2000), *Arbutus unedo* and *Taxus baccata* (Metaxas et al., 2004), *Ebenus cretica* (Syros et al., 2004), *Gardenia jasminoides* (Hatzilazarou et al., 2006) and *Malus* rootstock MM 106 (Naija et al., 2008). In our work the pattern of change in peroxidase activity was similar in the stem bases and leaves of IBA- leaf bud cuttings of elepidote rhododendron. We found typical changes of peroxidase activity in the induction and initiation phases, in accord with results previously described (Gaspar et al., 1994). However, our results suggest that not only reduction but also a gradual increase of peroxidase activity can be used to define the expression phase. This might be explained by the comparatively long period of time required for rooting in rhododendron cuttings. Anatomical data indicated that the first root initial formation took place in the initiation phase. Adventitious root initial formation from phloem rays has also been observed in *Ficus pumila* leaf bud cuttings (Davies et al., 1982). The next adventitious root initial formation and development of root primordia occurred in the expression phase simultaneously. Similar findings have been reported for microcuttings of *Malus* rootstock MM 106 (Naija et al., 2008) where initiation and development of adventitious roots were not synchronous and different stages of adventitious root development were observed at the same time in single pieces of stem.

Adventitious root formation did not occur in IBA+ leaf bud cuttings, so rooting phases could not be identified. Peroxidase activity in leaves of leaf bud cuttings did not show a well-defined maximum activity peak, but in cutting bases two peroxidase peaks were observed. The first peak of peroxidase activity might be due to an auxin concentration exceeding the optimum concentration for rooting rhododendron cv. 'Babites Baltais' leaf bud cuttings. Metabolic changes in the rooting zones of cuttings due to phytohormones/auxins are capable of either inhibiting or promoting adventitious root regeneration (Husen and Pal, 2007). As in many other responses to plant growth regulators, the effect of auxin on rooting is promotory at low concentrations and inhibitory at high (superoptimal) concentrations (de Klerk et al., 1997). Grönroos and Von Arnold (1987) reported that superoptimal IBA treatments extended the period for development of meristemoids and roots in hypocotyl cuttings of *Pinus contorta* in vitro, and the



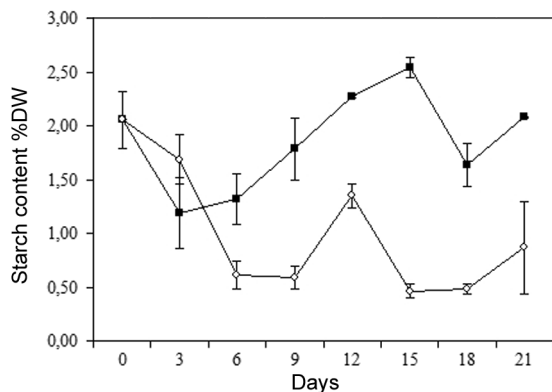
**Fig. 1.** Transverse section of the rooting zone of elepidote rhododendron cv. 'Babites Baltais' leaf-bud cutting grown without IBA. **(a)** Anatomical structures of stem base. sx – secondary xylem; ca – cambium; ph – phloem; sc – sclerenchyma fibers; co – cortex. Bar = 50  $\mu$ m, **(b)** Root initial (asterisk) formation from phloem ray cells (arrowheads) in 9-day-old cutting. Bar = 10  $\mu$ m, **(c)** Root primordia (asterisk) formation in IBA- 18-day-old cutting. Bar = 50  $\mu$ m.



**Fig. 2.** Change in peroxidase activity in IBA- **(a)** and IBA+ **(b)** leaf bud cuttings of elepidote rhododendron cv. 'Babites Baltais' during rhizogenesis. (■) activity in cutting bases, (◇) activity in cutting leaves. Values ( $\pm$  SE) are means of three determinations from three independent measurements for each date.

maximum number of roots was obtained more than a month after cutting. There is an inverse relationship between changes in auxin level and changes in specific peroxidase activity (Gaspar et al., 1994). High peroxidase activity in cuttings of the non-rooting genotype of *E. cretica* probably reduced the auxin level in tissues, which was a prerequisite for root formation to occur [soluble peroxidase activity was measured using 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-(dimethylamino) benzoic acid (DMAB)] (Syros et al., 2004). In our experiment as well, the first peak of peroxidase activity shows a sharp drop of auxin level in stem bases of leaf bud cuttings. The second peak of peroxidase activity in IBA+ leaf bud cuttings might be explained by intensive callus formation. Ludwig-Müller (2003) reported a peroxidase peak in *Grevillea rondeau* on day 25, when a large callus had grown [total peroxidase activity was measured using o-phenylenediamine (OPD) as substrate].

During the rooting process, starch is converted to soluble carbohydrate; in *Hydrangea*, starch disappeared from the endodermis, phloem, xylem rays and pith in tissue adjacent to the developing root primordia, and was converted to soluble carbohydrates (Hartmann et al., 2002). In this study the starch content in IBA- leaf bud cuttings decreased during the first few days, then gradually rose to maximum, followed by a sharp reduction and another increase at the end of the experiment. An initial decrease of starch content in the bases of cuttings



**Fig. 3.** Changes of starch content in bases of leaf bud cuttings of elepidote rhododendron cv. 'Babites Baltais' during rhizogenesis; (■) IBA- cuttings, (◇) IBA+ cuttings. Values ( $\pm$ SE) are means of three determinations from three independent measurements for each date.

has been demonstrated in *Pisum sativum* cuttings. This decrease may be due to closure of stomata, which diminishes the cutting's ability to fix CO<sub>2</sub> (Veierskov, 1988). Despite the formation of root initials, starch content kept increasing gradually. Ahkami et al. (2009) also noted that starch seems not to be involved in root initiation as only a marginal increase of starch occurs in early stages. We recorded decreased starch content in the bases of leaf bud cuttings during simultaneous formation of adventitious root initials and development of root primordia. As adventitious root formation is an energy-demanding process (Veierskov, 1988), starch stored in the rooting zone of cuttings is utilized to provide the energy needed for it (Husen and Pal, 2007). Histochemical investigations in IBA-treated *Pinus radiata* hypocotyl cuttings showed that starch began to build up preferentially in cells involved in or situated close to potential sites of new root formation before organized root primordia were visible, and began to disappear during root primordium formation (Li and Leung, 2000). Despite those demonstrated changes in starch content during rooting of cuttings, we observed that changes in starch content in IBA- leaf bud cutting bases did not coincide with the rooting phases as peroxidase activity did. This means that changes of starch content cannot be used as a rooting phase marker in rhododendrons. We did not observe any significant starch accumulation in bases of IBA+ leaf bud cuttings. Similarly, Haissig (1989) reported no starch accumulation in cuttings from sexually mature *Pinus banksiana* trees and no visible root formation by the end of the propagation period.

There was a significant correlation between peroxidase activity in leaves and bases of IBA- rhododendron leaf bud cuttings. We suggest that peroxi-

dase activity shows potential as a marker for rooting phases in leaves of rhododendron leaf bud cuttings. Further study of the relationship between the peroxidase activity and anatomical and physiological features during leaf bud cutting rooting is needed.

## ACKNOWLEDGEMENTS

We thank Inga Apine, Ineta Samsone and Una Andersone for technical assistance. The present work was supported by European Social Fund no. 2009/0138/1DP/1.1.2.1.2/09/IPIA/VIAA/004 and by the Latvian Council of Science (grant no. 04.1292, "Research of anatomical and physiological processes in woody plants during their vegetative propagation and their further development").

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