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Biochemical Status of Stock Plants and Their Annual Sprouts as a Crucial Key for Successful Adventitious Root Formation

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Abstract

Leafy cuttings of *Prunus subhirtella* Miq. 'Autumnalis' were harvested from mature, semi-mature and juvenile stock plants at four dates during the vegetative period 2011 (on 16th of May, on 30th of May, on 20th of June and on 11th of July) and their auxin levels (IAA, IAA-Asp) and sugar content (glucose, sucrose, sorbitol) were quantified. The IAA and IAA-Asp contents in cutting bases increased over the vegetative period, whereby aspartate values were higher than IAA values. The IAA-Asp values ranged from 6.3 μg g^{-1} to 22.7 μg g^{-1} FW and reached two great peaks on 30th of May and on 11th of July. The IAA values ranged from 0.29 μg g^{-1} to 4.51 μg g^{-1} FW, reaching a small fall on 20th of June. Significantly higher levels of IAA and IAA-Asp were measured at the base of mature cuttings compared to cuttings of semi-mature and juvenile origin, when the cuttings were harvested on 30th of May and on 11th of July. On the other hand, mature leafy cuttings accumulated significantly less fructose and glucose in their root emergence zone (16.3 g kg^{-1} DW, 45.2 g kg^{-1} DW) compared to semi-mature cuttings (26.4 g kg^{-1} DW, 62.5 g kg^{-1} DW) and juvenile cuttings (27.3 g kg^{-1} DW, 73.9 g kg^{-1} DW). All measured rooting parameters (rooting success, number of main roots and root length) were significantly improved when cuttings of a more juvenile origin (semi-mature and juvenile) were used.

Keywords: indole-3-acetic acid, N-(3-indolacetyl) aspartic acid, severance date, maturation, juvenility, rhizogenesis, carbohydrate content

Abbreviations: adventitious rooting = AR, adventitious root formation = ARF, indole-3-acetic acid = IAA, IAA-Aspartate = IAA-Asp

Introduction

Stock plant quality plays a very important role in the adventitious root formation (ARF) process. In herbaceous plants, optimal nutrition, irrigation and various horticultural measures are essential for achieving healthy and vigorous stock plants. However, in addition to the optimization of technological procedures, the physiological status of stock plants is crucial for ARF in woody plants (Hartmann *et al*., 1997; Spethmann, 1997; Osterc, 2009; Rasmussen and Hunt, 2010). An optimal physiological status of stock plants can significantly improve the rooting success of leafy cuttings (Rasmussen and Hunt, 2010). Nevertheless, the understanding of physiological ageing of woody plants remains fairly incomplete (Osterc, 2009) although it is tightly linked to economical results of the horticultural production (Dodd and Power, 1988; Spethmann, 1997). A very important segment in the understanding of woody plant maturation process is the possibility of reversing the mature plants into juvenile ones. *In vitro* propagation methods serve as an excellent possibility of generating physiologically juvenile plant material which is genetically identical as the original stock plant but rejuvenated in order to achieve optimal rooting success. ARF is namely significantly improved when juvenile instead of mature stock plant material is used for propagation of leafy cuttings in different woody plant species (Spethmann, 1997; Morgan and Williams, 1976; Plietsch and Heiliger, 1997; Osterc *et al*., 2009; Osterc and Štampar, 2011).

Biochemical research of adventitious rooting (AR) has been focused on determining the levels of indole-3-acetic acid (IAA), the main compound affecting adventitious rooting process. Several research studies have confirmed that IAA can affect the AR process either by endogenous IAA levels measured in cuttings at the time of severance or by induced IAA metabolism during the induction and initiation phase of AR (Osterc *et al*., 2009; Ford *et al*., 2001; Nag *et al*., 2001). Endogenous IAA level of severed cuttings is directly connected to stock plant ability to accumulate IAA (Osterc *et al*., 2009). As already known, environmental conditions play a crucial role, because only plants, grown under optimal conditions can accumulate enough endogenous IAA necessary for subsequent rooting (Hartmann *et al*., 1997). The physiological status of stock plants and their parts is also very important as juvenility/maturity greatly influences IAA accumulation in woody plants (Osterc, 2009). However, only a few studies stressed the importance of this parameter in the past. Osterc *et al.* (2009) measured IAA accumulation in root emergence zone of ornamental cherry leafy cuttings at the time of severance and determined significant differences among different physiological origin of the cuttings.

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Also, the accumulation of different sugars, affected by physiological status of stock plants, can be of potential interest. In woody plants, the involvement of sugars in the process of AR is a little controversial. High sugar contents quantified in annual shoots of mature and juvenile stock plants showed optimal photosynthesis and sufficient supply with nutrients. Therefore it cannot be concluded that carbohydrates have a regulatory role in rooting potential of leafy cuttings *per se* (Veierskov, 1988; Druege *et al.*, 2000; Druege, 2009).

The aim of this study was to determine the accumulation of IAA and IAA-Aspartate (IAA-Asp) in annual sprouts of stockplants during the vegetation period. The analysed areas on the sprouts were their bases because they represented zones of root induction process, when the cuttings were prepared. The question weather the intensity of auxin accumulation in annual shoots could be affected either by severance date or by different physiological status of the stock plants has been solved. Additionally, the content of sugars in annual shoots was measured at the severance point and their levels compared among of stock plants of different physiological status. This represents a new aspect of studying different backgrounds of root induction process including the physiological status of the material (sprouts) serving for cutting preparation. Therefore, this aspect can be an interesting chapter in adventitious root formation physiology in woody species.

Materials and methods

Plant material

Ornamental cherry (*Prunus subhirtella* Miq.) cultivar 'Autumnalis' leafy cuttings were harvested from different stockplants, distinguished by their physiological status. Three treatments were established: (1) mature plants, more than 40-year-old cherry plants; (2) semi-mature plants, 5-year-old plants propagated by leafy cuttings obtained from mature cherry plants; and (3) juvenile plants, 5-year-old *in vitro* derived plants (rejuvenated above mentioned mature cherry stock plants). All plants were located at the experimental field of Biotechnical Faculty in Ljubljana (Slovenia) to ensure equal environmental conditions.

Mature stock plants were rarely pruned (only dry branches), whereas semi-mature and juvenile stock plants were pruned every spring. Therefore, mature plants were up to 10 m height, semi-mature and juvenile plants were bushy, up to 1.5 m height. Leafy cuttings were harvested four times during the growing period: on 16th of May, 30th of May, 20th of June and 11th of July. The propagation material was always adjusted to 12 cm long leafy cuttings with apical meristems. On average, the cuttings had three to four fully developed leaves.

Cutting propagation

The cuttings were inserted in the substrate mixture of peat and sand (1:1) without any hormone treatment. Prior to insertion, the substrate mixture was fertilized with a slow-release fertilizer (2.0 g l^{-1} 3-4M Osmocote® Exact 16+11+11+3 Mg+Te; Scotts International, Heerlen, The Netherlands), and the pH value was adjusted to 4.0 with dolomitic lime. All experimental treatments (cuttings from mature, semi-mature and juvenile stock plant material) were replicated 3 times with 11 cuttings per plot (3 cuttings per plot for auxin analyses, 8 cuttings for rooting evaluation and sugar analyses).

The experiment was set in an unheated plastic house equipped with a fogging system (Plantfog-Befeuchtungsanlagen Nebelsysteme, Fishamend, Austria). The air temperatures in the plastic house reached up to 50 °C during daytime and ranged between 18 and 20 °C during the night. The substrate temperature (rooting zone) did not undergo such oscillations (between 20 and 24 °C), mainly due to a quality fogging system. Fogging was regulated manually to obtain 90-95% relative humidity. Fogging intervals lasted approx. 30 s, with a 60 s pause and were switched off during the night (19.00-07.00 h).

The evaluation of propagation success was always performed four weeks after severance. The number of successfully rooted leafy cuttings, and main roots were counted and the root bush length was measured.

Extraction and analyses of IAA and IAA-Asp

Leafy cuttings (three cuttings per treatment for each sampling date) for auxin analysis were collected on the day of severance and immediately transferred to laboratory facilities. All leaves were cut off and discarded and only the basal parts (root emergence zone, lower 3 cm of the cuttings) were used for further analysis. The samples were washed and stored at –20 °C until the analysis were performed.

Auxins were extracted following the methods of Štefančič *et al.* (2005), Kovač *et al.* (2003) and Goncalves *et al.* (2008). Samples were ground to a fine powder using a mortar and pestle with liquid nitrogen. Each sample was divided into two portions, 0.15 g each, and extracted separately with 1 ml BHT-MeOH solution [0.5 g of BHT (2,6-di-tert-butyl-4-methylphenol) per 1l solution] and 4 ml 5 mM potassium phosphate buffer, adjusted to pH 6.5. After 1 h at 4 °C, the extract was filtered and 3 ml 5 mM potassium phosphate buffer (pH value 6.5) was added to each sample. For auxins purification, Strata C18-E columns (pore size 55 μm, retention capacity 500 mg, tube size 6 ml; Phenomenex, Torrance, CA, USA) were used. The complete sample extracts were first run through the column pre-conditioned with potassium phosphate buffer (pH value 6.5) and then washed with 4 ml of 5 mM potassium phosphate buffer (pH value 6.5). The eluate was acidified to pH value 2.5 with 0.5 M H_3PO_4, then transferred to a second column pre-conditioned with potassium phosphate buffer (pH value 2.5). The column was rinsed with 2 ml twice-distilled water and eluted with 2 ml 80% (v/v) methanol.

The concentrated eluate (1 ml) was separated by TSP (Thermo Separation Products) HPLC using a Chromsep (Varian, Palo Alto, CA, USA) column [SS 250 × 4.6 mm, Hypersil 5 ODS (Octa Decyl Silica)] and analysed by a fluorescence (Spectrasystem FL2000; SpectraPhysics, San Jose, CA, USA) and a UV-VIS detector (K-2500; Knauer, Berlin, Germany). The mobile phase consisted of solvent A (acetonitrile/glacial acetic acid/twice-distilled water, 10/2/88, v/v) and solvent B (100% acetonitrile). The gradient was isocratic from 84% solvent A and 16% solvent B the first 20 min to 95% solvent A and 5% solvent B for the rest of the 30 min method, and the flow rate was 1 ml/min. Excitation of the fluorescence detector was set at 292 nm and emission at 360 nm. The UV-VIS detector was adjusted to measure absorption at 280 nm (Goncalves *et al.*, 2008). IAA and IAA-Asp were quantified by fluorimetry, comparing peak areas with those of corresponding standards. Losses were evaluated by standards that had passed through the extraction and purification processes.

Table 1. Rooting, number of main roots and root bush length of *Prunus subhirtella* 'Autumnalis' leafy cuttings of different physiological origin propagated on four severance dates

Severance date	Physiological status	Rooting (%)	Number of roots (n)	Length of roots (cm)
16th of May	Mature	100.0 ± 0 c	6.0 ± 2.3 ab	2.8 ± 1.2 abc
	Semi-mature	100.0 ± 0 c	20.3 ± 11.2 c	5.5 ± 1.2 cdef
	Juvenile	100.0 ± 0 c	25.7 ± 8.1 cd	6.9 ± 2.5 ef
30th of May	Mature	86.7 ± 11.5 c	6.0 ± 5.5 ab	2.3 ± 2.4 ab
	Semi-mature	100.0 ± 0 c	24.2 ± 5.6 cd	8.1 ± 1.5 f
	Juvenile	100.0 ± 0 c	29.4 ± 10.7 de	7.3 ± 1.6 ef
20th of June	Mature	26.7 ± 23.1 a	3.3 ± 0 a	3.5 ± 0 abcd
	Semi-mature	60.0 ± 20.0 b	3.6 ± 3.1 a	3.0 ± 1.1 abc
	Juvenile	60.0 ± 20.0 b	11.3 ± 3.5 b	7.8 ± 2.8 f
11th of July	Mature	48.9 ± 25.2 ab	4.1 ± 4.2 ab	1.7 ± 1.2 a
	Semi-mature	100.0 ± 0 c	19.5 ± 6.9 c	4.9 ± 2.3 bcde
	Juvenile	95.8 ± 7.2 c	33.2 ± 7.5 e	6.1 ± 1.5 def

Means ± standard error are shown. Different letters assign significant differences for each set of dates individually at $P<=0.05$, Duncan test

Auxins were identified using a mass spectrometer (Thermo Scientific, LCQ Deca XP MAX) with an atmospheric-pressure chemical ionization (APCI) operating in positive ion mode. The analyses were carried out using full scan data-dependent MS^n scanning from *m/z* 50 to 1000. The injection volume was 10 µl and the flow rate maintained at 1.0 ml min^{-1}. The capillary temperature was 275 °C, the sheath gas and auxiliary gas were 35 and 10 units respectively; and the source voltage was 16 kV. Spectral data were elaborated using the Excalibur software (Thermo Scientific). The identification of compounds was confirmed by comparing retention times and their spectra as well as by adding the standard solution to the sample and by fragmentation. Calculated auxin contents (IAA and IAA-Asp) of each sample (n=3) were the means of two replicate measurements and were expressed in µg g^{-1} FW.

Sugar analyses

Individual sugar contents (glucose, fructose and sorbitol) were measured in leafy cutting root emergence zone at the day of severance and samples were prepared as described previously for auxin analyses. Samples were lyophilised and between 0.3 g and 2.0 g material was ground to a fine powder using mortar and pestle. Plant material was immersed in 3 to 20 ml of twice-distilled water (depending on the amount of plant material used) and left for extraction for 30 min at room temperature with frequent stirring. The extracted samples were centrifuged at 10000 × g_n for 7 min at 10 °C (Eppendorf Centrifuge 5810R, Hamburg, Germany). The supernatant was filtered through a 0.45 µm filter (Macherey-Nagel), transferred to a vial and stored at -20 °C until analysis by high-performance liquid chromatography (HPLC; Thermo Scientific, Finnigan Spectra System, Waltham, MA, USA). For each analysis, 20 µl of sample was used. The analysis of sugars was carried out using a Rezex-RCM-monosaccharide column (300 × 7.8 mm; Phenomenex, Torrance, CA) with a flow rate of 0.6 ml/min and column temperature maintained at 65 °C. For the mobile phase, twice-distilled water was used, and an RI (refractive index) detector for identification. The identification and quantification of individual carbohydrates were calculated with the addition of corresponding external standards and expressed in g kg^{-1} DW.

Statistical analyses

Statistical analyses were carried out with the Statgraphics Plus (version 4.0) programme, using analyses of variance (ANOVA). Statistically significant differences between treatments (date of severance and physiological status) were tested with the Duncan test at a 0.95 confidence level.

Results

Rooting

Rooting success was evaluated four weeks after insertion into the substrate. Cuttings obtained from mature cherry stock plants rooted significant poorer than more juvenile cuttings on all severance dates, except on 16th of May, when the rooting was 100% in all treatments. Rooting results clearly indicate that the difference in rooting percentage between mature cuttings and rejuvenated cuttings (by propagation or *in vitro* rejuvenation) increased at later severance dates (Table 1). Root development is a clear factor of successful rooting and cuttings from rejuvenated stock plants (semi-mature and juvenile) developed more roots, which were also longer compared to cuttings obtained from mature stock plants (Table 1). Up to 30 roots with a length exceeding 8 cm were visible on semi-mature and juvenile cuttings and there were significant differences among different severance dates (Table 1).

IAA and IAA-Asp

The levels of free IAA in cutting bases ranged from 0.29 to 4.51 µg g^{-1} FW during the growing season. The IAA values slightly increased over the season, with expect on 20th of June, when a little fall was noticed. The aspartate levels in cutting bases reached in general higher levels than IAA and ranged from 6.3 to 22.7 µg g^{-1} FW. The levels increased over the season with two great peaks, on 30th of May and on 11th of July (Fig. 1). Unexpectedly high levels of IAA were quantified in the root emergence zone of mature leafy cuttings. The values ranged between 0.23 and 12.72 µg g^{-1} FW in mature cuttings and were significantly higher on the 30th of May and on the 11th of July compared to cuttings obtained from semi-mature and juvenile stock plants (Fig. 2). The IAA-Asp profile was very similar to that of IAA. Again, the base of cuttings severed from mature cherry stock plants contained more aspartate compared to more juvenile cuttings at all sampling dates. IAA-Asp profile reached two peaks, on 30th of May and on 11th of July when the concentrations of 41.3 and 56.8 µg g^{-1} FW were significantly higher than those measured in semi-mature and juvenile cuttings at the same time (Fig. 3).

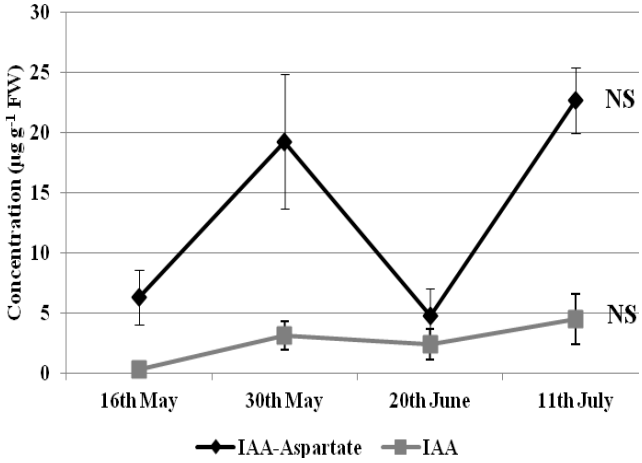

Fig. 1. IAA and IAA-Aspartate level at the base of *Prunus subhirtella* 'Autumnalis' leafy cuttings on different severance dates during the growing season 2011. Means ± standard error are shown. NS assigns no significant differences at $P<=0.05$, Duncan test

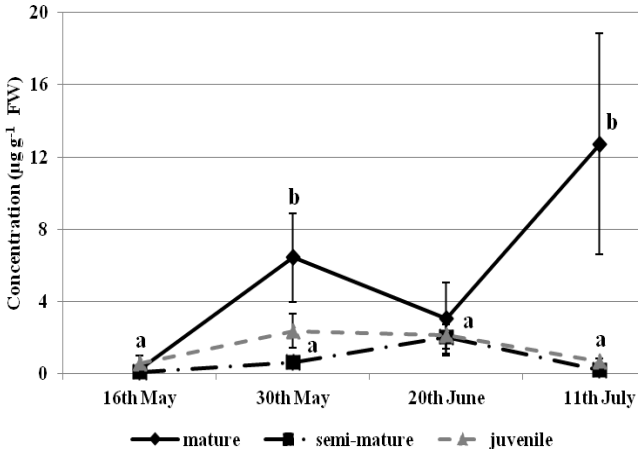

Fig. 2. IAA content level at the base of *Prunus subhirtella* 'Autumnalis' leafy cuttings of different physiological origin on four severance dates. Means ± standard error are shown. Different letters assign significant differences for each set of dates individually at $P<=0.05$, Duncan test

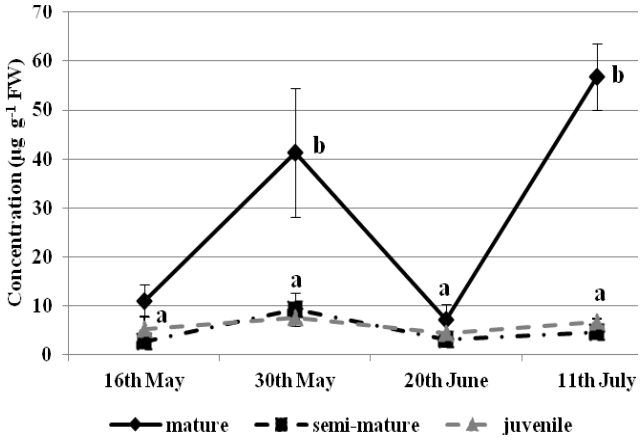

Fig. 3. IAA-Aspartate content level at the base of *Prunus subhirtella* 'Autumnalis' leafy cuttings of different physiological origin on four severance dates. Means ± standard error are shown. Different letters assign significant differences for each set of dates individually at $P<=0.05$, Duncan test

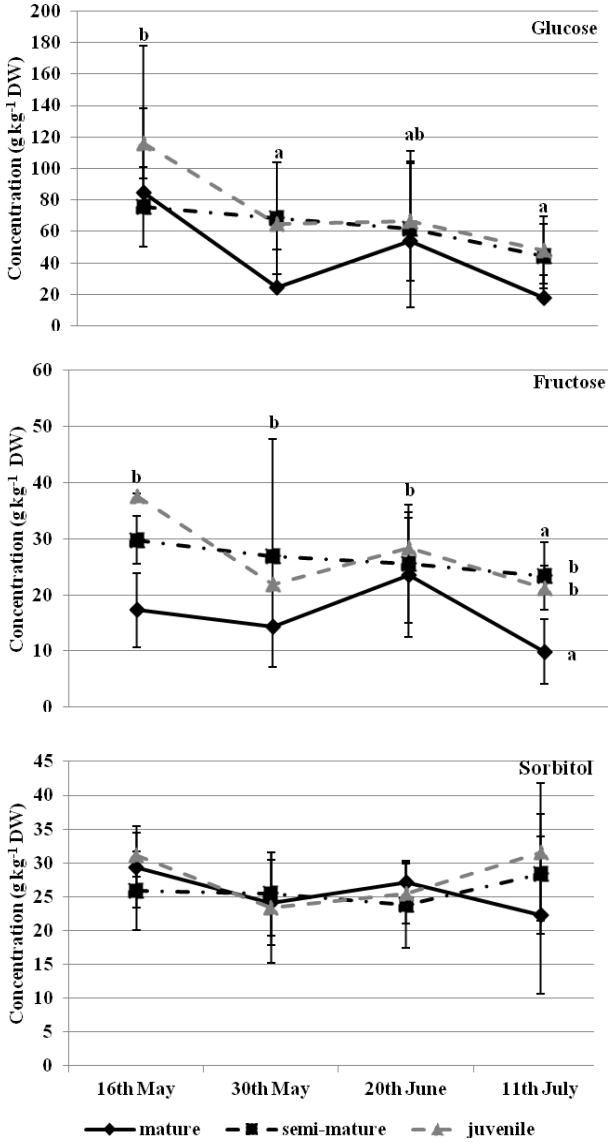

Fig. 4. Content level of different sugars at the base of *Prunus subhirtella* 'Autumnalis' leafy cuttings of different physiological origin on four severance dates. Means ± standard error are shown. Different letters assign significant differences for each set of dates individually at $P<=0.05$, Duncan test

Sugars

Glucose and fructose content levels measured at the base of ornamental cherry leafy cuttings decreased during the vegetative period, whereas sorbitol content was similar during the whole analysed period. Glucose levels decreased from 92.1 g kg^{-1} DW at 16th of May to 36.7 g kg^{-1} DW at 11th of July. Fructose content was even lower and a decrease from 28.2 g kg^{-1} DW in the middle of May to 18.2 g kg^{-1} DW in the middle of July was measured. Fructose content levels also differed among different physiological origin of the cuttings. Cuttings from mature cherry stock plants accumulated significantly less fructose (16.3 g kg^{-1} DW) compared to cuttings severed from semi-mature (26.4 g kg^{-1} DW) and juvenile stock plants (27.3 g kg^{-1} DW). A similar glucose profile was established among treatments but the differences were not significant (Fig. 4).

Discussion

The rooting results obtained in this study clearly support well known data on great differences in rooting success between mature and juvenile cutting material from numerous previous reports (Spethmann, 1997; Morgan and Williams, 1976; Plietsch and Heiliger, 1997; Osterc *et al.*, 2009). An excellent rooting result of ornamental cherry juvenile cuttings was recorded regardless on the severance date. On the other hand, mature cuttings rooted well only on the first severance date and a decreased rooting ability was noted later in the vegetative period. The number of roots also showed the same trend. Rooting profile of mature cuttings was similar to the rooting profile of specific difficult-to-root cuttings reported in some previous studies (Ford *et al.*, 2001). Therefore, it can be concluded that the importance of the optimal rooting conditions for successfully rooting increase strongly with the maturation status of stock plants. According to our results, semi-mature cuttings reacted similarly as juvenile cuttings and their rooting capacity was less affected by severance date.

Nevertheless, horticultural and forestry history deals with many experiments, which stress the importance of optimal severance date for subsequent rooting in numerous plant species (Spethmann, 1997; Ford *et al.*, 2001; Osterc *et al.*, 2007). Ford *et al.* (2001) showed that the percent transport of IAA, the intensity and the velocity of hormone transport declined over the growing season. The decline was in this experiment especially strong in lilac, classified as difficult-to-root species. Spethmann (1997) could show in his experiments that lilac leafy cuttings could be rooted with the highest ratio at the beginning of the growing season, immediately before flowering. In our experiment the IAA concentrations in cutting bases slightly increased over the growing season (except on 20th of June) but together with the IAA also the concentrations of IAA-Asp increased. The rooting results were in average the highest at the beginning of the growing season, when the IAA concentration was at the lowest level, but also the concentration of IAA-Asp, which is signed as IAA inactivation compound (Bartel *et al.*, 2001), was also at the lowest level at the same time.

Differences in rooting success were often explained with genetic variation among species. Ford *et al.* (2001) categorised lilac as a difficult-to-root species and *Forsythia* as an easy-to-root genus. Osterc *et al.* (2007) ascribed oscillations in rooting response and callus formation between two chestnut cultivars to intraspecific clone differences. However, biochemical patterns, characteristic of difficult- and easy-to-root species, have rarely been studied. Ford *et al.* (2001) ascribed differences in rooting success of lilac and *Forsythia* to differences in IAA transport ability to annual shoots. Moreover, Osterc *et al.* (2007) quantified various polyphenolic compounds in chestnut cuttings and indicated that differences exist among cultivars and also among different severance dates, but the differences were not always significant.

In the present study, however, auxin levels (IAA and IAA-Asp content) differed significantly between mature cuttings and cuttings obtained from more juvenile ornamental cherry plants on all sampling dates. Similarly, differences in rooting success were measured among treatments with the exception of 20th of June. At that time both IAA content levels and rooting response of leafy cuttings of all three physiological types were similar.

The accumulation of high levels of IAA and IAA-Asp was unexpected in mature cuttings especially in relation to lower auxin levels quantified in juvenile material which rooted better. Based on the findings of Ford *et al.* (2001), great rooting differences between mature and more juvenile cuttings in our experiment cannot be explained by a weaker polar IAA-transport in mature cuttings. The IAA over-accumulation in mature cuttings led to poorer rooting due to the inhibitory effect of high IAA content levels on ARF. A similar reaction has already been reported in herbaceous plants (Faivre-Rampant *et al.*, 2002), but in woody plants it has not been demonstrated previously. Faivre-Rampant *et al.* (2002) explained poor rooting results of tobacco *rac* shoots with the inhibitory effect of the supra-optimal concentration of active auxin at the time of AR. Marks *et al.* (2002) reported a potential stress reaction of difficult-to-root woody species (such as lilac) which transported exogenously applied IAA with greater intensity compared to easier-to-root *Forsythia* cuttings. Štefančič *et al.* (2005) also stressed the importance of the optimal IAA content levels in the rooting material during the first hours after severance in order to achieve ideal subsequent rooting. Cherry cultivars with supra-IAA content levels namely showed poorer rooting potential. Moreover, different biochemical compounds may trigger various regulatory mechanisms in woody plants. Osterc *et al.* (2008) reported higher content levels of various polyphenolic compounds in chestnut cuttings and linked them to a better rooting potential. The type of a regulatory mechanism of the specific compound depends on the type of function which this compound has in a plant. Over-accumulation of aspartat in the mature cuttings at the time of severance indicates that a large part of auxin remains inactive. This can be explained by high levels of IAA-Asp, which can potentially inactivate IAA activity (Bartel *et al.*, 2001) and thus directly influencing the rooting success.

Carbohydrates (glucose and fructose) serve as a nutritional storage and thus a higher content levels measured in juvenile cuttings could be linked to their better rooting ability. Higher levels of sugars namely boost the development of roots, which consequently represent a stronger sink for different nutrients. Druege *et al.* (2000) demonstrated that a higher sucrose: starch ratio in leaves of chrysanthemum cuttings at the time of severance increased rooting at specific severance dates. On the other hand, Druege (2009) also stressed that the initial carbohydrate reserves in chrysanthemum cuttings are less important for subsequent rooting when the photosynthesis of cuttings after inserting in the substrate is sufficient for normal plant and root development. Based on some previous data, it can be assumed, that juvenile cuttings are able to photosynthesise on much higher levels compared to mature cuttings (Teiz and Zeiger, 2006). Higher initial carbohydrate reserves and a higher level of photosynthesis of juvenile cuttings both represent a sufficient nutrient pool for successful subsequent rooting.

Conclusions

The correct time of severance of specific plant species and cultivars is closely correlated with the endogenous auxin level in cutting material. Understanding the regulatory effect of the endogenous IAA level in mature cuttings led us to conclude that the optimal level of endogenous IAA at the time of severance is of crucial importance for subsequent rooting

potential. Beside IAA level also levels of other conjugates, especially those who inactivate the IAA action (like IAA-Asp) is of great importance. The quantification of the ideal IAA level of specific propagation material is surely the next step to take. Answers to these questions can only be elucidated by monitoring different plant groups and their physiological status which is the work of our future.

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