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Initial Cutting Length Modifies Polyphenol Profile in *Castanea* Cuttings during the Root Formation Process

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Summary

The issues of cutting length have not been discussed seriously in cutting propagation history. As the results from practice show, cutting lengths between 5 and 15 cm have proven optimal – woody species, as well. On the basis of the newly developed fog systems (high pressure fog systems), the use of very long cuttings (more than 50 cm) became possible for the first time. The results using these longer cuttings from various woody species were surprisingly good. However, the experiments with long cuttings have never been applied to chestnut (*Castanea* sp.). With a rate of over 20 %, the rooting percentage of long cuttings was much better in comparison to short ones. The leaves of long cuttings accumulated significantly more rutin (>0.35 mg g^{-1} FW), quercetin-3-O-rhamnoside (quercitrin; >0.38 mg g^{-1} FW), quercetin-3-D-galactoside (hyperoside; up to 2.80 mg g^{-1} FW), and quercetin-3-D-glucoside (0.28 mg g^{-1} FW), but also more ellagic acid (up to 0.13 mg g^{-1} FW). The differences between long and short cuttings were the greatest at the time of severance; later the differences decreased.

Key words. leafy cutting – fog system – adventitious rooting – *Castanea crenata*×*Castanea sativa*

Introduction

During recent years, the leafy cutting propagation method has undergone much improvement, particular in woody plants. The initial cutting length seems to be a very important factor, which can significantly affect rooting results. Some years ago, mostly standard cutting lengths between 5 and 20 cm were used in various plant species. However, several experiments carried out in recent years have opened the discussion about very long cuttings having a strong rooting potential (Spethmann and Huhn 2001; Mbabu and Spethmann, 2005; Spethmann 2007). Of course, the employment of long cuttings was generally made possible owing to recent efficient developments in fog systems (Spethmann, 1997). Since this is a completely new technology that has never been applied until now, very little is known about the quality of such cuttings, but this can be crucial for rooting success. Štefančič et al. (2007) reported that the stress following insertion into the substrate could be largely reduced in long *Castanea* cuttings by using a fogging atmosphere in the propagation house. But in the same study, similar behaviour could also be observed in short cuttings, indicating that the cutting quality should be investigated as well.

Regarding cutting quality, polyphenol accumulation in short cuttings immediately after insertion into the substrate was often studied. Several results indicated that even in woody species some polyphenol compounds act as rooting cofactors in the root formation process. Chlorogenic acid, ellagic acid and some flavonoids (rutin, quercetin-3-D-galactoside) are described as rooting cofactor compounds in *Castanea* cuttings (Osterc et al., 2007, 2008). In herbaceous plants too, chlorogenic acid was reported to play an important role in the enhancement of the rooting process (Faivre-Rampant et al. 2002). In *Prunus* cuttings, phloroglucinol and vanillic acid were also ascribed a stimulatory effect during the rooting process (Trobec et al., 2005).

In chestnut (*Castanea* sp.), long cuttings have never been used. It is well known in practice that chestnut growth after the propagation season is often problematic. Consequently, the growing period necessary to grow plants ready for sale can be spread over several years. The use of cuttings with greater initial lengths can probably overcome this problem, because higher plants can be produced as early as during the propagation season. In this study, we compared short and long cuttings regarding their polyphenol status. The aim was to investigate the fluctuations of various polyphenol substances in cuttings during root induction and the root initiation phase. The comparison was carried out with chestnut cuttings, which have been recorded as difficult-to-root in several studies (Breisch, 2000; Osterc et al., 2007, 2008). The conclusions reached might be used for future research with other woody species.

Material and Methods

Experimental design and plant material

The experiment was carried out in an unheated plastic house (up to 50 °C during daytime and between 18 and 20 °C during the night) with a high-pressure fogging sys-

tem at the Biotechnical Faculty of Ljubljana University (Slovenia) in 2004. The fogging system assured constant high humidity in the house (90–95 %), with bursts of fine fog lasting 30 s emitted every 90 s during the day. Fogging was stopped during the night (19:00–7:00). The rooting medium (3:1 wet peat:sand with 2 g l^{-1} substrate slow-released fertilisers [Osmocote® Exact 16+11+11+3Mg+Te]) did not exceed 24 °C during the experiment. The pH-value of the substrate was adjusted to 4.0 with 0.9 g lime l^{-1} substrate. The experiments started on 10th June; the periods between bursts of fog were extended on 15th August and were completely stopped on 30th September.

On 10th June, 256 cuttings were taken from 6-year-old, annually pruned, field grown plants (the stock plant care followed the nursery standards) These were derived from an *in vitro* culture and grown at the Faculty Experimental Station in Maribor (Slovenia). Approximately 50 shoots (50–80 cm long) per stock plant were removed. The cuttings of two *Castanea crenata×Castanea sativa* hybrid clones, 'Marsol' (clone CA07) and 'Maraval' (clone CA74), were used in the experiment. Terminal cuttings of two different lengths (12 and 50 cm) (5–9 mm diameter) were prepared for both clones (4–6 leaves per short cutting and 12–15 leaves per long cutting). Each treatment was represented by 32 cuttings×4 replicates (n=128). Each cutting was dipped in 0.5 % IBA (with 10 % 'Euparen® WG 50'(Bayer CropScience AG Monheim, Germany) in a talcum base) before insertion in the rooting substrate (peat:sand in a 3:1 ratio). The cuttings remained in the substrate until the final rooting assessment at the end of the growing season. On 15th November 2004, the percentage of cuttings rooted, and callus formation were evaluated.

Extraction and analysis of polyphenol compounds

Each day of sampling, 2 cuttings per replicate were included in the polyphenol analysis (every day for 7 days and on day 14 and 21 after severance, one full-developed leaf from each cutting was used per replicate).

For the polyphenol analyses, samples (0.20 g) were extracted with 5 ml methanol containing BHT (1 %; 10 g 2,6 di-tetra-butyl-4-methylphenol-BHT/1l methanol) in an ultrasonic bath for 45 min. After the extraction, the solvent was centrifuged and filtered through membranes (MN, Chromafil 25 mm, 0.45 μm pore size) and stored at −20 °C until the high performance liquid chromatography (HPLC) analyses.

Polyphenols were determined with reversed phase HPLC of Thermo Separation Products (TSP) with the detector WellChrom K-2500 and Thermo Finigan Surveyor and PDA detector, Varian Chromsep column (SS 250×4.6 mm, Hypersil 5 ODS) and OS/2 Warp IBM and Chrom Quest 4.0 Operating System. The mobile phase consisted of solvent A (water - acetic acid, 1 %) and solvent B (methanol:acetonitrile; 1:1, v/v) with the following gradient elution: 0–10 min, 15 % B; 10–50 min, 15–40 % B in A; 50–60 min, 40–60 % B in A; 60–80 min, 60–100 % B in A (Fernandez-Lorenzo et al., 1999) with a flow rate of 0.5 ml min^{-1}. The injection volume was 5 μl. The individual phenolic compounds were identified and quantified by comparing their retention time and UV (absorbance) spectra with those of reference standards.

Statistical analysis

Statistical analyses were carried out with the Statgraphics Plus statistical program (version 4.0, Manugistics, Statistical Graphics Corp.) using the analysis of variance (ANOVA). Statistically significant differences among the treatments were tested with the Duncan test at a 0.95 confidence level.

Results

The initial cutting length showed the strongest effect on rooting results, because, at a rate of 23.5 %, longer cuttings rooted significantly better than the short cuttings with a rooting rate of just under 1 %. Nearly 8 % of the rooted long cuttings formed calluses, whereas all rooted short cuttings also developed calluses (Fig. 1).

Various flavonols were analysed in chestnut leaves. The highest amounts, nearly 3.0 mg g^{-1} FW, were measured in the case of quercetin-3-D-galactoside (hyperoside); this was followed by nearly 0.40 mg g^{-1} FW of quercetin-3-O-rhamnoside (quercitrin), 0.35 mg g^{-1} FW of quercetin-3-D-glucoside and 0.32 mg g^{-1} FW of rutin. The concentrations of all substances showed a significant difference with regard to the initial cutting length at the time of severance when the leaves of long cuttings contained significantly higher amounts of all substances than the leaves of short ones (Fig. 2).

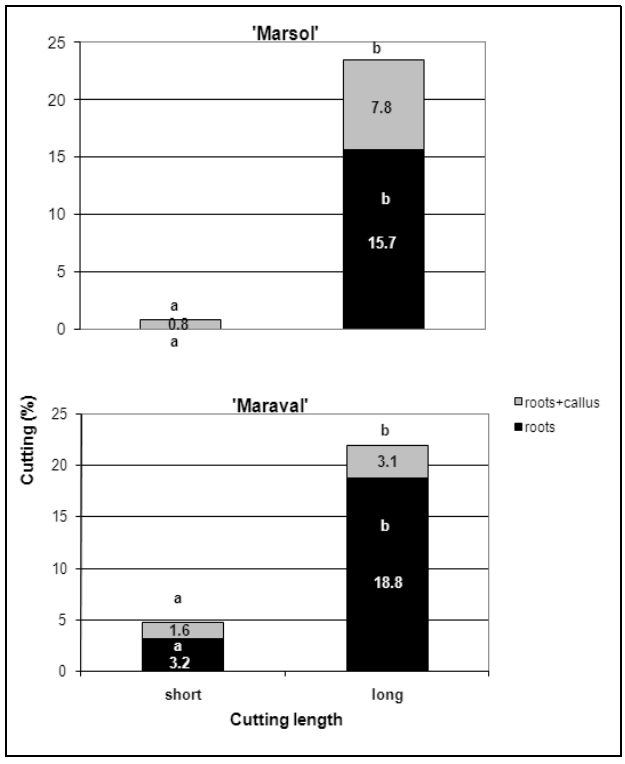


Fig. 1. Root and callus formation at 'Marsol' and 'Maraval' cuttings at the end of propagation season 2004 dependant on initial cutting length. Different letters indicate significant differences (P=0.05; Duncan test) between treatments.

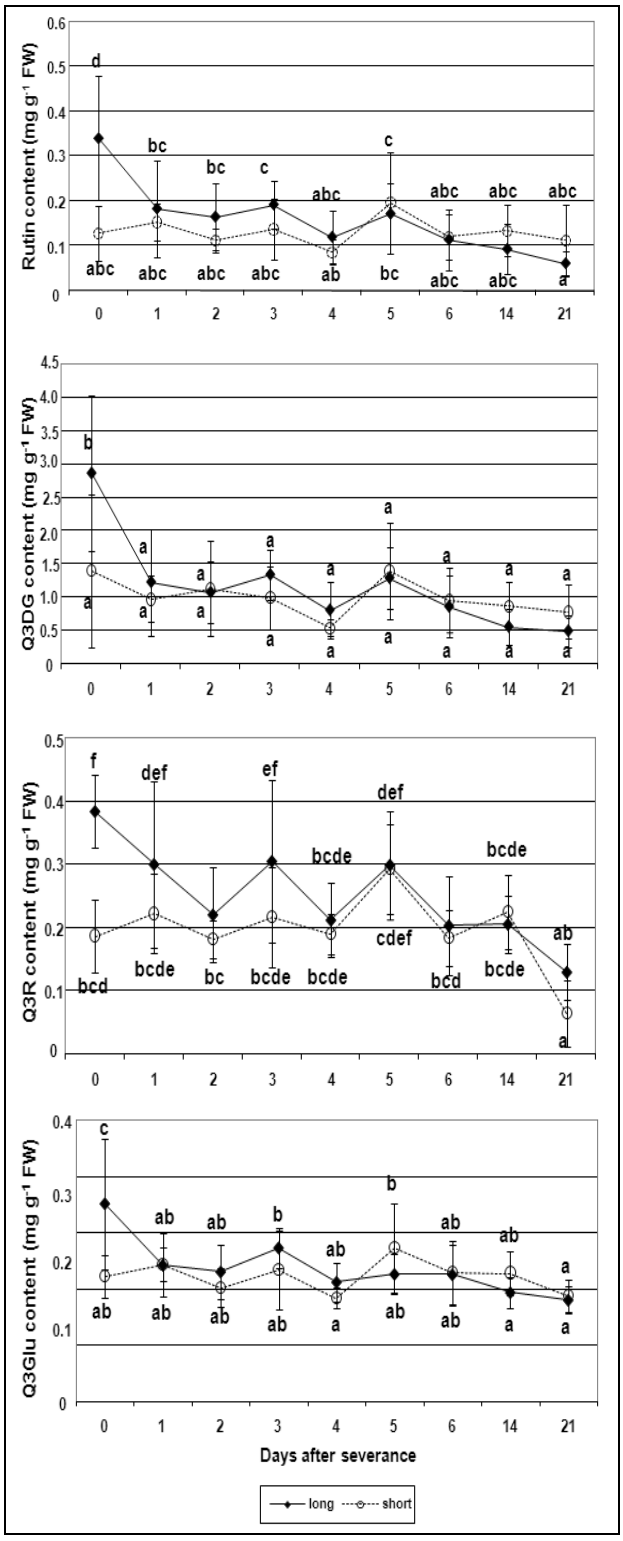


Fig. 2. Concentrations of different flavonols in cutting leaves during 21 days after severance dependant on initial cutting length. Means (SE) are shown together for both clones 'Marsol' and 'Maraval'. Different letters indicate significant differences (P=0.05; Duncan test) between treatments.

Phenolic acids were measured in leaves from the cuttings. The chlorogenic acid content levels reached

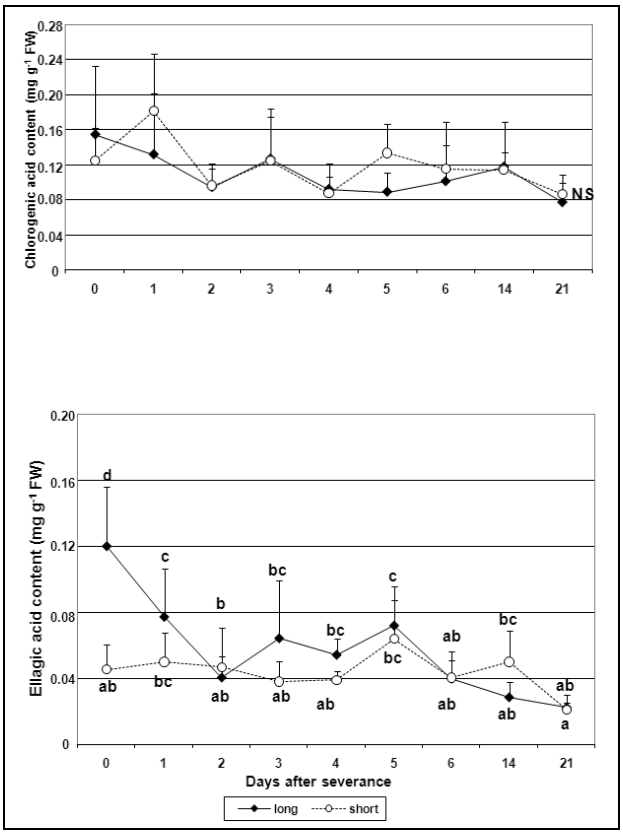


Fig. 3. Concentrations of ellagic and chlorogenic acid in cutting leaves during 21 days after severance dependant on initial cutting length. Means (SE) are shown together for both clones 'Marsol' and 'Maraval'. Different letters indicate significant differences (P= 0.05; Duncan test) between treatments.

0.18 mg g^{-1} FW, whereas contents of ellagic acid remained at 0.12 mg g^{-1} FW. The initial ellagic acid concentrations in the leaves of long cuttings were statistically more significant than those from the short cuttings. The chlorogenic acid values showed no statistically significant differences (Fig. 3).

Discussion

Several studies in recent years have stressed the importance of extremely long cuttings in the propagation technology of various woody species. The benefit of these cuttings could be shown in several production phases. The use of long cuttings could reduce the production time in *Rosa* sp. to one half (Spethmann and Huhn, 2001). Mbabu and Spethmann (2005) received better rooting with long *Pyrus* cuttings (100 cm) than with shorter ones (50 or 75 cm). In this study, the positional effect (topophysis) played an additionally important role. These results are also supported by our present study. The long cuttings rooted significantly better than the short ones. There are no studies available on the physiological aspects of long cuttings, because they represent a new approach in leafy cutting strategies. Some results from the past can be summarised. Spellerberg (1985, 1986) showed that sufficiently nourished cuttings grew better in the propagation season and survived the first winter period better. Mac

Carthaigh and Eble (1989) also stressed the importance of cutting nutrition for their subsequent growth and survival. It can be assumed that, owing to their length, the long cuttings are ready for better accumulation of mineral compounds than the short ones. Accumulation of photosynthetic products is probably significantly better in longer cuttings. According to various research data (Spethmann, 1997), this kind of accumulation also affects better cutting growth and survival.

Based on the results from Osterc et al. (2007, 2008), the accumulation of various polyphenols is crucial for the successful rooting of cuttings. Among them, various flavonols, rutin, quercetin-3-O-rhamnoside and quercetin-3-D-galactoside are important, but chlorogenic and ellagic acid also showed a significant effect on rooting. Chlorogenic acid was also in some other studies correlated with rooting success (Hartman et al. 1997, Faivre-Rampant et al. 2002). Faivre-Rampant et al. (2002) explained that chlorogenic acid serves in tobacco mutants as an oxidation medium for IAA-oxydase instead of IAA. Higher amounts of this phenolic substance mean in this way more IAA. These data are supported by our findings: leaves of long cuttings, especially at the time of severance, contained significantly more rutin, Q3R, Q3DG and ellagic acid. The time of severance is crucial for cuttings in view of the subsequent rooting process. The cutting quality (the level of IAA and different polyphenols) at the time of its setting into the rooting media is decisive for rooting success. The preparation of cuttings during the period between cutting removal from the stock plant and their setting into the rooting media has no significant influence on cutting quality. Some new data support this assumption. Štefančič et al. (2006) reported that exogenous IBA application increased the IAA content in comparison to a control treatment in a 'Gisela 5' cutting, but the rooting success was similar in both treatments. The main conclusion from this study was that the exogenous activity after the removal of cuttings from the stock plant is less important for the propagation results than their quality at the time of removal. This conclusion correlates with some of our previous results (unpublished data) with cuttings of ornamental cherry species. Juvenile cuttings rooted significantly better than adult cuttings, owing to higher IAA content levels in their bases at the time of severance. Their bases also contained more chlorogenic acid and rutin in this period.

Other studies have also contributed the decisive role of cutting quality at the time of severance. Harbage et al. (1998) reported that the rooting response of some apple microcuttings was significantly better in rooting media with low pH-value, owing to better absorption of IAA from rooting media in acid conditions. The identical response was also found under in-vivo conditions. The leafy cuttings (lengths between 10 and 15 cm) of different plant genera (*Quercus*, *Prunus*) rooted significantly better in substrate mixtures with pH-values ranging from 3.5 to 4.0 (Spethmann, 1997). The behaviour of long cuttings is the opposite, as reported by Mbabu and Spethmann (2005). The long cuttings tended to show better rooting response in substrate mixtures of greater pH-values (>6.8). It can be concluded that longer cuttings do contain, at the time of severance, more naturally formed IAA and they do not need the exogenous stimulus.

The present study upgraded some new applicative studies about the positive role of long cuttings. We have demonstrated, on the basis of some phenolic compounds that are involved in the rooting process that the major impact of cutting length lies in better cutting quality at the time of severance. Further experiments, especially with auxin metabolism depending on initial cutting length are, of course, obligatory to confirm these results.

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