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Changes in Polyphenols in Leafy Cuttings During the Root Initiation Phase Regarding Various Cutting Types at *Castanea*

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With 3 Figures

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Summary

OSTERC G., TROBEC M., USENIK V., SOLAR A. & ŠTAMPAR F. 2004. Changes in polyphenols in leafy cuttings during the root initiation phase regarding various cutting types at *Castanea*. – Phyton (Horn, Austria) 44 (1): 109–119, with 3 figures. – English with German summary.

There are some disadvantages of the present propagation methods (grafting, micropropagation) of the genus *Castanea* (*Fagaceae*). The leafy cutting propagation method, which seems to be the best alternative, is to be optimised to achieve successful propagation results. The polyphenol analyses during the root initiation phase and the root growing phase (first month after being put in the substrate) were carried out to clarify some problems during the rooting process (bad rooting, strong callus formation). Several polyphenol substances which were identified in the cuttings, can negatively affect the rooting process. The tannic and gallic acid were identified in cutting bases and stems. In cutting leaves tannic acid, gallic acid, ellagic acid, quercetin-3-d-galactoside (hyperoside, Q3DG) and quercetin-3-rhamnoside (quercitrin, Q3R) were identified. The contents of all those substances increased over the period from the time the cuttings were put in the substrate till the fifth day in the substrate. The increase of tannic acid and quercetin-3-rhamnoside after five days determined in cutting leaves was significant.

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Zusammenfassung

Osterc G., Trobec M., Usenik V., Solar A. & Štampar F. 2004. Polyphenoldynamik während der Wurzelentstehungsphase in Abhängigkeit von verschiedenen Stecklingsarten bei den *Castanea* Grünstecklingen. – Phyton (Horn, Austria) 44 (1): 109–119, 3 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Die gebräuchlichen Vermehrungsmethoden (Veredlung, Mikrovermehrung) bei der Gattung *Castanea* (*Fagaceae*) haben bestimmte Nachteile. Die Vermehrung mit Grünstecklingen, die die beste Alternative zu sein scheint, soll optimiert werden, um erfolgreiche Vermehrungsergebnisse zu erzielen. Es wurde die Analyse der Polyphenole während der Wurzelbildungsphase und der Wurzelwachstumsphase (erster Monat nach dem Stecken ins Substrat) durchgeführt, um einige Probleme während der Wurzelentstehungsphase (niedrige Bewurzelungsraten, starke Kallusbildung) zu klären. Einige Polyphenole, die in den Stecklingen gefunden wurden, könnten sich negativ auf den Bewurzelungsprozess auswirken. Tannin- und Gallussäure konnten in der Basis und den Stielen der Stecklinge festgestellt werden. In den Blättern der Stecklinge wurde Tannin-, Gallus- und Eleagnussäure, Quercetin-3-d-Galaktosid (Hyperosid, Q3DG) und Quercetin-3-Rhamnosid (Quercitrin, Q3R) gefunden. Die Werte all dieser Substanzen nahmen bis zum fünften Tag nach dem Stecken zu. Die Zunahme der Tanninsäure und des Quercetin-3-Rhamnosid in den Blättern der Stecklinge nach fünf Tagen konnte statistisch gesichert werden.

Abbreviations: quercetin-3-d-galactoside (hyperoside) = Q3DG, quercetin-3-rhamnoside (quercitrin) = Q3R, FW = fresh weight.

Introduction

There are certain disadvantages of both commonly used propagation methods, grafting and micropropagation, in *Castanea* species. When applying grafting methods there is a possibility of the infection by some pathogens (*Cryphonectria parasitica, Erwinia amylovora*). The in-vitro methods are very expensive, the growth of plants is often not homogenous enough and a lot of time is needed to produce plants, which are ready for sale (Spethmann 1997, Osterc & Spethmann 2000). The real chance to propagate chestnut plants economically on their own roots seems to be achieved with the leafy cutting propagation method. Osterc & al. 2001 succeeded to achieve with leafy cuttings of the *Castanea* hybrid clones 'Marsol' and 'Maraval' the average rooting 15–20% but the method requires to be improved before used in the praxis.

Numerous factors are present which can affect the rooting process. Beside auxins which act as direct rooting stimuli through affecting RNA (Hartmann & al. 1997, De Klerk & al. 1999), a main role was ascribed to polyphenols (Haffner & al. 1991, Hartmann & al. 1997, De Klerk & al. 1999, Kling & Meyer 1983, Curir & al. 1993) due to their synergistic or inhibitory effects on the rooting process (Hartmann & al. 1997). Among synergistic ones especially (iso)chlorogenic acid and catechol were mentioned (Hartmann & al. 1997). Catechol was also considered a synergistic

substance during the rooting process (KLING & MEYER 1983). MAYNARD & BASSUK 1990 reported about the inhibitory effect of catechol on the rooting process in their experiment. Discrepancies were observed also when studying the effect of tannic acid. Whereas STILL & al. 1976 found an inhibitory effect of tannic acid on the rooting process, KLING & MEYER 1983 observed a positive effect of this compound on the root formation. DE KLERK & al. 1999 regarded ferulic acid as an IAA-synergistic factor. Anyway, some of those studies were not comprehensive enough, because they deal only with one concentration of a compound (DE KLERK & al. 1999). Consequently, the results describing the effects of various compounds differed greatly.

It seems the role of the phenolic compounds in the process of the root formation have to be assessed through the involving of these compounds in the IAA metabolism during this period. For the regulation of IAA level in the plants and in this way also for the effect of IAA on root initiation the IAA oxidative catabolism is very important. IAA oxidative catabolism means the loss of the auxin activity, and it is the only irreversible output regulating IAA levels. There are two different IAA catabolism pathways in plants: decarboxylation pathway and oxindole-3-acetic acid/dioxindole-3-acetic acid pathway. The last pathway has been identified in at least three plant species, although it would be assumed that this pathway is widely distributed (DAVIES 1995). Anyway, at this point this pathway is not so important. More important seems to be the decarboxylation pathway which is decisive better understood as the oxindole-3-acetic acid/dioxindole-3-acetic acid pathway. It is catalysed through peroxidase and the main products of this reaction are 3-methylene-oxindole, 3-hydroxymethyloxindole (oxindole-3-carbinol), indole-3-aldehyde, and indole-3-methanol. Phenolic compunds can regulate this reaction and they regulate throughout also the IAA level in the plants. FAIVRE-RAMPANT & al. 2002 underlined the importance of this role of phenolic compound with tobacco plants. The chlorogenic acid acted at tobacco plants as the main inhibitor of IAA oxydation and caused throughout the strong auxin accumulation which had the inhibitory effect on root initiation. Generally, monophenols and m-diphenols stimulate IAA oxydation, while p-diphenols, o-diphenols, coumarins, and polyphenols inhibit the enzyme reaction (DAVIES 1995, FAIVRE-RAMPANT & al. 2002).

Additionally, we still know very little about what actually happens in the first period after the cuttings are put in the rooting substrate. The hypothesis of HARTMANN & al. 1997 is very well known and stresses the active role of the added auxins only four days after the cutting. The hypothesis was supported also by later histological experiments (DE KLERK & al. 1999). We have very little information about the types of polyphenols and their role in the phase of root formation. The knowledge about the poly-

phenol action together with auxin in the rooting initiation phase is especially weak in the case of woody plants. Among them very little information are collected for leafy cuttings, because the studies which have already been conducted, have examined in vitro plants and not leafy cuttings. In addition, little do we know about the activities in the *Castanea* genus occurring after the cuttings are put in the substrate. These are the questions which we want to clarify with the present experiment.

Material and Methods

Experimental Design and Plant Material

The experiment was carried out in an unheated plastic house under a fog system at the experimental field of the Biotechnical Faculty in Ljubljana (Slovenia) in 2001. Two different cutting types (terminal and basal cuttings) were tested in a experiment with 4 replications and 40 cuttings per replication with the *Castanea* hybrid clone 'Marsol' (*Castanea crenata* × *Castanea sativa*). Cuttings were cut on 13th June at the Faculty experimental station in Maribor (Slovenia); five-year-old in-vitro plants (in-vitro derived plants) were used as the mother plants. Before being put in the rooting substrate (peat/sand in a 3:1 ratio) cuttings were treated with 0.5% IBA (with 10% Euparen on talcum basis). For the propagation 12 cm long cuttings were used.

Extraction and Analysis of Phenolic Compounds

The material for polyphenol analyses was collected on the cutting day and after the cuttings were placed in the substrate and remained there for one, five, nine, 14, and 29 days. The cuttings gathered for further polyphenol analyses were divided into base part (2 cm), stem part and leaves (Fig. 1). All segments were immediately frozen in liquid nitrogen.

For polyphenol analyses the samples were extracted with acetone-water (80:20, v/v) containing Triton X-100 (0.4%) for 30 min. or 10 days (base and stem parts) at 4 °C (USENIK & ŠTAMPAR 2000, FERNANDEZ-LORENZO & al. 1999). In a mortar 100 mg of plant material was homogenised with 20 ml of extraction solution. After extraction, the solvent was evaporated at 40 °C. The residue was dissolved in methanol (2 ml). The samples were clarified and filtered through membranes (MN, Chromafil 25 mm, 0.45 µm pore size). The samples were stored at –20°C until HPLC analyses.

Polyphenols were determined with reversed phase HPLC of Thermo Separation Products (TSP). The HPLC equipment consisted of X-ACT^{TM} deggaser, P2000 TSP pump, Chromsep SS (250 × 4.6 mm) Hypersil 5 ODS, reversed phase column, guard column, Chromsep Guard SS (10 × 3 mm) reversed phase, autosampler AS 1000, detector WellChrom K-2500 for detection at 280 nm (UV), and OS/2 Warp IBM Operating System (1994). The mobile phase was dependent on the material which was analysed. For leaves analyses 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 & al. 1999). For analyses of the stem parts and bases the solvent A (water – phosphoric acid, 0.05%) and solvent B (acetonitrile, 100%) with the following gradient elution: 0–40 min., 10% B; 40–

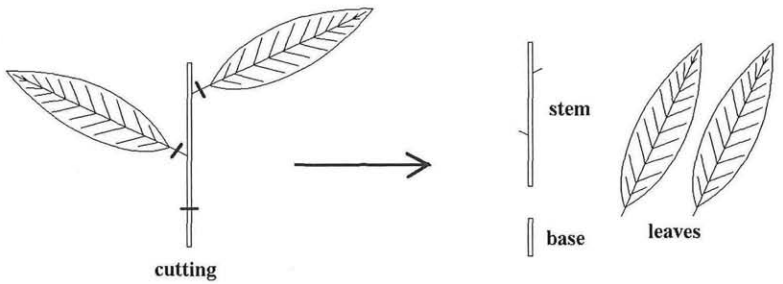

Fig. 1. Division of cuttings for polyphenol analyses

60 min., 10–50% B in A; 60–65 min., 50–100% B in A were used (GROSS 1994). In both cases the flow rate was 0.5 ml/min and the injection volume was 5 µl.

The individual phenolic compounds were tentatively identified comparing their retention time and UV spectra with those of authentic samples.

Statistical Analyses

The experiment was evaluated as two-factor experiment (cutting type, date of analysis) with ANOVA, the analyses of averages were tested with the Duncan-test at $p = 0.05$. For evaluation the statistic program Statgraphics Plus 2.1 was used.

Results

Propagation Results

Generally, rooting response of the cuttings was very bad. The rooting percentage was lower than 1%. This result stresses clearly, how deep is the propagation problematic at *Castanea* genus. The survival of the cuttings was associated with the callus formation. The cuttings survived and formed up to 5% callus in the propagation period (Fig. 2).

Polyphenols in Stems and Bases

In cutting stems and bases tannic and gallic acids were identified. Terminal cuttings contained higher concentration of both substances than basal cuttings but that was not the case for gallic acid in cutting bases (Table 1).

Cutting bases contained on average more tannic acid than the stems. The tannic acid content curves of the terminal and basal cuttings differed greatly of each other during the first month of the cuttings being in the substrate. The tannic acid content of terminal cuttings, measured in both bases and stems decreased over the period of one month after they had been placed in the substrate. The tannic acid content in basal cuttings measured in bases and stems increased in this period. The tannic acid

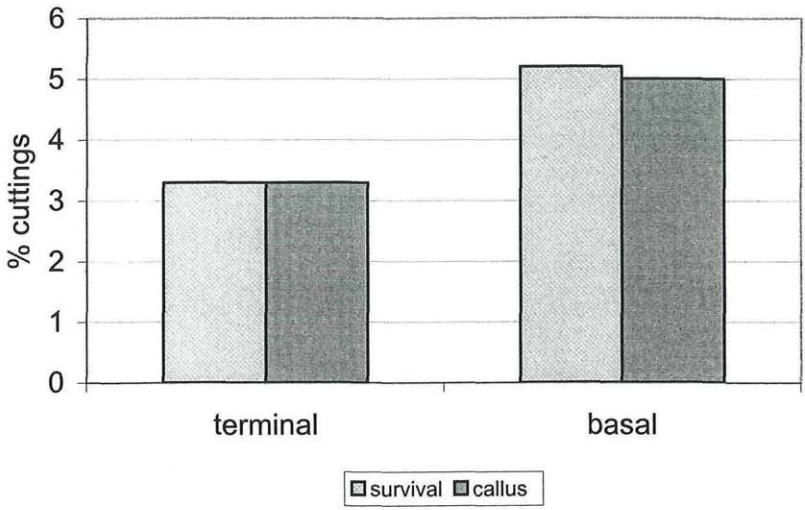

Fig. 2. Survival and callus formation at cuttings of Castanea hybrid clone 'Marsol' regarding different types of cuttings

content varied significantly along the days and in cutting bases the interaction between the date of analysis and a type of cutting was confirmed. The differences between basal and terminal cuttings and the dates of analyses were significant in tannic acid measured in cutting stems (Table 1).

The differences in the gallic content between terminal and basal cuttings were not so clear, although in most cases the terminal cuttings contained higher amounts of the gallic acid than the basal ones. There was a high decrease in gallic acid in the bases of terminal cuttings at the first day in the substrate and then the content remained on the same level over the period of one month. The decrease could be observed also in gallic content in stems of basal cuttings whereby this fall was not so high. The gallic amounts in stems of terminal cuttings increased slowly between the first day and the day 9 after the cuttings had been placed in the substrate. The differences between both types of cuttings when measured in stems could be indicated as significant. The differences between the time remaining in the substrate were significant when gallic acid was measured in bases. (Table 1).

Polyphenols in Leaves

In the leaves we determined 4 different substances: tannic and ellagic acids, quercetin-3-d-galactoside (hyperoside, Q3DG) and quercetin-3-rhamnoside (quercitrin, Q3R).

Table 1. Tannic and gallic acids content in base and stem parts of two different types of cuttings at different days of analyses of 'Marsol' leafy cuttings. Means signed with different letters are significantly different (Duncan, p≤(0.05), * statistical analysis was not possible.

Type of cutting	Days in substrate	TANNIC ACID (mg/g FW)		GALLIC ACID (mg/g FW)	
		bases	stems	bases	stems
Terminal	0*	94.1	43.2	7.050	0.518
	1	77.5 bc	31.2	0.330	0.345
	5	83.3 c	28.2	0.086	0.641
	9	44.4 abc	21.7	0.004	0.983
	14	48.9 abc	23.1	0.682	0.602
	29	31.6 a	22.4	1.954	0.900
	Average	57.1	25.3 b	0.611	0.724 b
Basal	0*	20.5	15.7	0.786	1.539
	1	34.2 a	25.4	0.439	0.286
	5	36.4 ab	24.0	0.449	0.639
	9	24.8 a	20.2	0.004	0.427
	14	22.6 a	20.3	1.020	0.626
	29	25.3 a	20.1	1.335	0.432
	Average	28.6	22.0 a	0.650	0.450 a

Table 2. Ellagic acid, quercetin-3D-galactoside and quercetin-3-rhamnoside content in leaves of two different types of cuttings at different days of analyses of 'Marsol' leafy cuttings. Means signed with different letters are significantly different (Duncan, p≤(0.05), * statistical analysis was not possible.

Type of cutting	Days in substrate	ELLAGIC ACID (mg/g FW)	Q3DG (mg/g FW)	Q3R (mg/g FW)
Terminal	0*	0.53	2.11	2.44
	1	0.91 ab	2.31 ab	3.55
	5	1.56 b	3.63 b	3.96
	9	1.03 ab	1.73 a	3.01
	14	1.28 ab	1.65 a	3.59
	29	0.77 ab	0.93 a	2.13
	Average	1.11	2.05	3.25 b
Basal	0*	0.42	1.79	1.50
	1	0.60 a	1.82 a	2.51
	5	0.77 ab	1.85 a	2.68
	9	1.06 ab	1.68 a	2.46
	14	0.68 ab	1.02 a	2.14
	29	0.85 ab	0.98 a	2.15
	Average	0.79	1.47	2.14 a

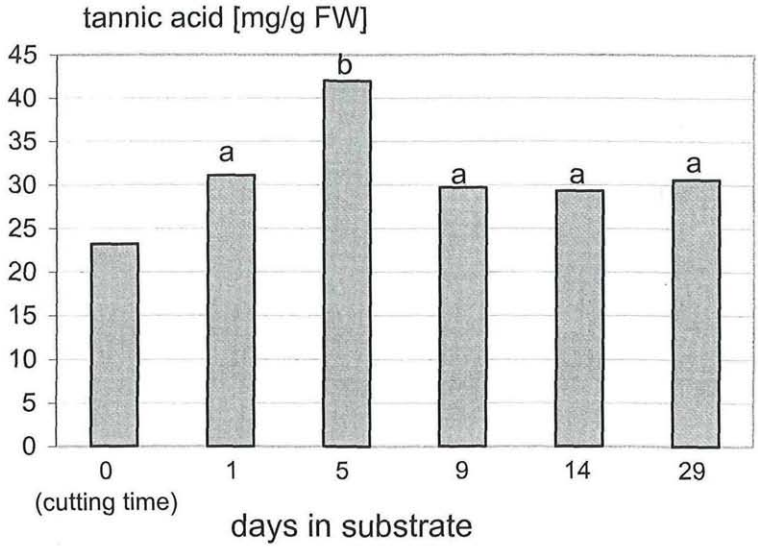

Fig. 3. Tannic acid content in cutting leaves during the first month remaining in the substrate in Castanea hybrid clone 'Marsol'. Means signed with different letters are significantly different (Duncan, p(0.05)

In general, the concentration of all substances determined in the leaves was similar, both measured in terminal and basal cuttings during one month in the substrate. An increase in tannic acid (Fig. 3) and Q3R (Table 2) was significant after five days of remaining in the substrate.

In most cases the leaves of terminal cuttings contained higher concentrations of the substances than the basal cuttings did. Especially visible is the difference in the case of Q3R, where the alteration was significant (Table 2).

When comparing the dates of analyses and types of cutting several interactions could be observed. In the leaves of terminal cuttings the highest amounts of ellagic acid and Q3DG were detected on the fifth day after being placed in the substrate. The lowest values of ellagic acid were measured in the leaves of the basal cuttings on the first day after the cutting, whereas the concentration of Q3DG was the lowest in the leaves of terminal cuttings after one month (Table 2).

Discussion

Ellagic and tannic acids are known as substances which inhibit the rooting process and react antagonistically with the auxin in the first steps after the cuttings are put in the substrate (KLING & MEYER 1983). These reports can be support by the outcomes of our research. The basal cuttings

which showed a better rooting reaction (stronger callus formation) contained significantly lower amounts of tannic and ellagic acids. The difference in tannic acid which could be measured in all plant parts (bases, stems and leaves) was especially evident in cutting bases. HARTMANN & al. 1997 thought about the cutting base as the most important place for the auxin presentation if the rooting process is required, although it should be mentioned that the added hormon with the mass-flow very quickly reached all the parts of the cutting (OSTERC & SPETHMANN 2001). Therefore it may be that also the effect of a rooting inhibitory factor is the strongest at the place where the external auxin is present at the beginning – this is at the cutting bases. The high tannic acid values at cutting bases at *Castanea* in our experiment can mean stronger formation of condensed tannins (barriers for root growth) and therefore in generally troubles in rooting process. This explain numerous reports about *Castanea* as extremely difficult-to-root species (FERNANDEZ-LORENZO & al. 1999, SPETHMANN 1997).

The first four days were regarded as the period when the presence of auxin is necessary for the root formation (HARTMANN & al. 1997, DE KLERK & al. 1999). HARTMANN & al. 1997 considered this period to be the auxin active phase. From the rooting point of view, the presence of the phenolic substances, which inhibit the rooting process in the first days after being put in the substrate (auxin active phase) is not desired. This assumption is supported also by the results of our research where the polyphenol substances increased in the period of five days (auxin active phase) in basal cuttings. Later decreased polyphenol substances over the period of one month during which the cuttings remained in the substrate. This type of cutting showed a better rooting reaction than terminal cuttings did. FAIVRE-RAMPANT & al. 2002 considered that the level of phenolic compounds undergoes a time-course variation that exactly parallels that of free IAA. When these compounds have the inhibitory effect on free IAA level, can this negatively act on root initiation. This correlation show also our results as mentioned above.

Both polyphenol substances quercetin-3-d-galactoside (hyperoside) and quercetin-3-rhamnoside (quercitrin) which belong to the group of flavonoids were present in the leaves in very low concentrations. The descriptions of the role of this polyphenol group in the rooting process are contradictory. HAFFNER & al. 1991 marked them as substances which hinder the rooting process. PACHECO & al. 2002 described the effect of Q3R to be positive for the rooting process. In the research carried out by our group the basal cuttings, which showed stronger rooting reaction, contained in general lower amounts of Q3R than the terminal ones.

The gallic acid is considered to be a precursor of more complex polyphenols, especially soluble tannins. Several authors also stated that gallic acid acts in esterification of 1,2,3,4,6-penta-O-galloyl-β-D-glucose which

is the immediate and common precursor of the gallotannins and ellagitannins (SCALBERT & al. 1988, GROSS 1994). Additionally, in the literature the polyphenols (also gallic acid as a tri-phenol, gallotannins and ellagitannins) are mentioned as inhibitors of IAA-oxidase which helps to destroy IAA, so the presence of polyphenols should mantain the level of IAA (DAVIES 1995). In some cases the level of IAA can increase in this way so strong that the rooting process is inhibited. This correlation is well known for non-woody plants (FAIVRE-RAMPANT & al. 2002) but can be also assumed for woody plants. The content of gallic acid in our study was very low in cutting bases and stems. In cutting bases the high amounts of gallic acid were observed in the terminal cuttings before being put in the substrate, later already after one day a strong fall was detected. The lack of the callus and rooting reaction in the terminal cuttings can be probably due to these high amounts of gallic acid at the time of the insertion of the cuttings in the substrate. Gallic acid acted negatively on the rooting initiation and its amount decreased in the cutting bases.

We can conclude that there are several indicators which point to the fact that the propagation of chestnut with leafy cuttings is possible. The results showed certain effects of the cutting type on the rooting capacity. This effect could be well explained with the involvement of different polyphenol substances. Several previous hypotheses of the cooperation between some polyphenols and auxin during the root initiation (HARTMANN & al. 1997, HAFFNER & al. 1991, MATO & al. 1994, DAVIES 1995, FAIVRE-RAMPANT & al. 2002) proved to be correct. However, the future optimisation of the method is necessary. Studies which encompass the research into the optimal time of cutting and the formation of different polyphenol groups in different plant stages will lead us to some important and valuable conclusions in the future. Very perspective results can probably bring also the measurements of the endogene auxin levels in the correlation with the course of polyphenol substances.

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