University of West Hungary

Theses of doctoral (Ph.D.) dissertation

The role of chemical parameters in the red heartwood formation of beech (*Fagus sylvatica* L.)

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1. Introduction and aims of the research

Facultatively coloured heartwood formation is the most important structural and colour anomaly of living beech, degrading significantly the value of beech stands, therefore causing substantial economic loss. Although the technical features and applicability of red heartwood differ only slightly from the parameters of non-discoloured beech wood, its usability in interior design, decorative and other visible applications is rather limited. Red heart is considered primarily as an aesthetical error of beechwood.

This makes the recognition of red-heartwooded stems and the determination of the rate of red heartwood formation in particular stands important. Several efforts have been made in this field e.g. by developing non-destructive indication methods, or by applying mathematical modelling and statistics to determine the probability of future red heartwood formation in a stem, by knowing its specific parameters like diameter, age, number of injuries etc. . Yet utilizable methods have still to be sought for.

The reasons for the formation of red heart in beech, its physiology and the chemical structure of the heartwood chromophores are only partly revealed. The processes of red heartwood formation in beech are most commonly explained by analogies, based on the research results of fundamentally different, but known discolouration processes of woody tissues. Regarding the chemical processes taking place in beech tissues, exactly identified molecular carriers and well defined reaction equations could only be established in the case of dissoluble carbohydrates. Nevertheless, the role of the dissoluble carbohydrates is only indirect, these compounds could only act as precursors to colouring substances. These facts make it difficult to forestry sciences and practice aiming to fight back red heartwood formation to develop appropiate methods and to widen

the spectra of industrial utilization of red-heartwooded beech (e.g. by colour homogenization).

The aim of the author was to study the chemistry of red heartwood formation in beech by carrying out accurate chemical analytical measurements in order to identify the molecular carriers and to unveil the chemical processes of red heartwood formation.

The concrete aims of research were as follows:

- To investigate the chemical environment of the tissue matrix in which the reactions and physiological processes of red heartwood formation take place. To measure the radial and longitudinal variation of the moisture content, pH, bound-, free-, and total acid content and buffercapacity of the tissues.
- To identify the role of oxidoreductase enzymes, especially that of peroxidase (POD) and polyphenol-oxidase (PPO) in the physiology of red heartwood formation. Scientific literature has not revealed yet the role of POD and PPO enzymes in the physiology of beech, although POD's role is already proven in the heartwood forming processes of obligatory coloured heartwood forming species and in the discolouration of plant tissues.
- To prove that the formation of the red heart is not caused by wood rot fungi.
- To analyze the radial and longitudinal variations of the total phenol content and the role of phenolic compounds in red heartwood formation. To isolate and identify phenolic compounds from beechwood, investigate their radial and longitudinal distribution in the stem. To identify the compounds taking part in the formation of the colouring substances.

- To model *in vitro* the reactions which yield the colouring substances of the red heartwood. Parallel investigation of the reactions with and without enzymes.
- To investigate and compare the products isolated from the red heart of beech and the products gained from the *in vitro* reactions.
- To compare the processes of red heartwood formation with those of *Juglans*- and *Robinia*-type obligatory coloured heartwood formation.

The author compared the results obtained from the investigation of red-heartwooded stems with the data measured from the same number of non-discoloured stems originating from the same forest districts, in order to track the differences and this way to be able to identify unequivocally the chemical characteristics of red heartwood formation. The measurements were carried out for severals years and were repeated on a large number of beech stems originating from stands with different climatic conditions.

In the interpretation of his research results the author also considered the data found in the literature for the heartwood formation processes of obligatory coloured heartwood forming species.

2. Experimental methods

2.1 Sample collection and processing

For his measurements the author took a large number of disks which had been taken from the stems from a height of three meters. Three red-heartwooded and three non-red-heartwooded beech stems were chosen from each forest stand.

In order to track the vertical variation of the chemical parameters, disks were taken from one red-heartwooded and one white stem, from the felling surface up to the first branch (fork) by a sampling interval of one meter.

In order to investigate the radial variation of the measured chemical parameteres eight samples, ranging from the bark to the pith were taken from the red-heartwooded disks and five samples from the non-discoloured disks. The comparability of disks with different diameter was achieved by assigning the samples in a given ratio of the disk radius.

The sample disks originated from various forest stands of SEFAG Ltd. (Kaposvár district) and TAEG Ltd. (Sopron district). The time of sample disk collection was chosen in the time range between 1999-2005 considering the seasonal period of obligatory coloured heartwood formation (july-january).

Milled wood samples were extracted according to the current method. Extraction was carried out using (1) water, (2) sodium-acetate or (3) 4:1 methanol:water solution.

2.2 Measured parameters and analytical methods

pH and acidity. The determination of the pH and acidity (free-, bound- and total acidity content) was achieved applying potentiometry (NÉMETH, 1987).

Activity of POD enzyme. The activity of POD enzyme was determined spectrophotometrically using 3,3'-diaminobensidine as substrate, measuring absorbance at 480 nm, according to the method of SHANNON et al. (1966).

Activity of PPO enzyme. The activity of PPO enzyme was determined spectrophotometrically using pirocatechin as substrate, measuring absorbance at 420 nm.

Determination of total phenol content. According to the method of Folin-Ciocâltău (SINGLETON and ROSSI, 1965), standard: quercetin.

Qualitative and quantitative analysis of phenolic compounds. The identification and quantitative analysis of *catechins* was achieved using thin layer chromatography. Stationary phase: TLC silicagel; Mobil phase: 9:1 diisopropyl-ether:formic-acid (FECKA et al., 2001); Chamber: normal unsaturated twin trough chamber; Visualization: spraying with vanillin + sulphuric-acid reagent (STAHL, 1962). Qualitative and quantitative evaluation: Camag TLC Scanner 3 densitometer, absorbance mode.

Identification and quantitative analysis of *quercetin, taxifolin* and their *glycosides* was achieved using optimum performance laminar chromatography (OPLC). Stationary phase: HPTLC silicagel; Consecutive development with 6:3:1 toluene:ethyl-acetate:formic-acid and with 5:3:1:1 ethyl-acetate:methyl-ethyl-ketone:formic-acid:water mobile phases (STAHL, 1962) in OPLC 50 overpressurized chamber; Visualization: spraying with diphenylboric-acid-β-aminoethyl-ester and polyethylene-glycol-4000 solutions (STAHL, 1962). Qualitative and quantitative evaluation: Camag TLC Scanner 3 densitometer, fluorescence mode.

***In vitro* reaction of beech enzyme extract with beech phenols.** Reaction of beech enzyme extract and the identified phenolic compounds in aqueous solution at a pH characteristic to red-heartwooded tissues (pH=6.2). Separation of the compounds of the reaction solutions by thin-layer chromatography using the suitable mobile phases and TLC silicagel stationary phase. Quantitative and qualitative (UV-VIS reflectance spectra) evaluation of the products using scanning densitometry (Camag TLC Scanner 3).

Chemical characterisation of red heartwood chromophores. Extraction of heartwood substances with 4:1 methanol:water solution. Separation of the extracted compounds by thin-layer chromatography, using the suitable mobile phases and TLC silicagel stationary phase. Detection of the UV-VIS reflectance spectra of the products using scanning densitometry (Camag TLC Scanner 3).

Qualitative and quantitative analysis of sugars. Using optimum performance laminar chromatography (OPLC). Stationary phase: HPTLC silicagel; Mobile phase: 85:15 acetonitrile:water (SÁRDI et al., 1996); Development: in OPLC 50 overpressurized chamber with 2 × 6000 µl mobile phase (double development); Visualization: spraying with 4 g diphenyl-amin + 4 ml anilin + 20 ml 86% phosphoric-acid reagent. Quantitative evaluation: Camag TLC Scanner 3 densitometer, at 540 nm, absorbance mode.

Determination of total dissoluble carbohydrate content. Measured spectrophotometrically according to the method of DUBOIS (1956).

Imaging of the tissues with electron microscope. Investigation of the structure of beech wood tissues using raster electronmicroscopy (REM).

3. Methods of data processing and evaluation

The author used Microsoft Excel software for the evaluation of his research data. SPSS and Statistica 6.1 softwares were used for carrying out ANOVA analyses. The Tukey HSD calculation method was used, at p=0.05% significance level in all of the variance analyses. Microsoft Word word processor was used for the documentation.

4. Summary of scientific results (theses)

4.1 The author was the first to prove that oxidoreductase enzymes, i.e. POD and PPO have significant role in the physiological processes of red heartwood formation in beech.

- He investigated the pH dependence of the activity of POD and PPO enzymes. He revealed that at the pH values of red heartwood (6.1-6.8) PPO is specifically more active than POD.
- The author established that the activity of both of the enzymes increase sharply at the colour boundary and also remain at a high level in the inner heartwood tissues. This experience differs fundamentally from the known fact that absolutelly no enzyme actvity can be detected in the heartwood of obligatory coloured heartwood froming species.
- The author established that the ratio of the activity of POD and PPO enzymes is constant in all types of the woody tissues in beech, also including red heartwood. From these findings he concluded that the two enzymatic functions are in close connection with each other: probably the same isoenzymes are responsible for both of the enzymatic functions just like in *Quercus*-species.
- The author proved that the high enzyme activity detected in red-heartwooded tissues is not due to the presence of fungi. This confirmed those earlier research findings, which had concluded that red heartwood formation is a result of physiological processes.

4.2 The author isolated five phenoloid-glycosides and four phenoloid compounds from the woody tissues of red-heartwooded beech. Out of the isolated molecules (+)-catechin,

(−)-epicatechin, taxifolin and quercetin have been identified. After hydrolizing the glycosides quercetin and taxifolin have been evidenced as the aglycone of the glycosides.

- It has been evidenced that the concentrations of the five glycosides are high in the sapwood tissues. Moving on towards the inner woody tissues the concentrations decrease in both red-heartwooded and not-red-heartwooded stems.
- The glycosides hydrolize in the tissues at colour boundary – similarly as in obligatory coloured heartwood forming trees – and only the free aglycones can be detected in the tissues of the inner red heartwood.
- The colouring substances are mainly formed from the catechin epimers. The catechins' concentrantions are at a high level in front of the colour boundary and decrease sharply beyond that.
- In the discoloured wood tissues free taxifolin and quercetin accumulate. Their role in forming the chromophores is little.

4.3 The author modelled the formation reactions of the chromophores of the red heartwood by *in vitro* experiments. He parallely investigated the reaction of the four phenoloids with and without beech enzyme extracts.

- He evidenced that at the pH values of the red heartwood, (+)-catechin and (−)-epicatechin are transformed by the beech enzyme extract with molecular oxygen in oxidation and consecutive polymerisation reactions. The reactions also take place without the enzyme extract, but at a much lower rate and the production of the polymers is less significant.
- The author revealed that the transformation of taxifolin under the same conditions also takes place, but at a very low rate. The reaction can also be evidenced without the enzyme extract but the colour of the product is different in this

reaction. The oxidative conversion of taxifolin is significantly slower compared to that of the catechins.

- Quercetin was not transformed at all with the beech enzyme extract at the given pH and no coloured products could be detected.

4.4 The author compared the chemical features of the products gained from the *in vitro* reactions with those of the substances extracted from the red heart of beech.

- He proved that the chromophopres of the red heartwood are mainly the oxidized and polymerized products of (+)-catechin and (−)-epicatechin.
- Because of their slow reactions taxifolin and quercetin accumulate in the tissues of the red heartwood.
- It can be assumed that the four different phenoloids and their oxidation products also react with each other. The colouring substance of the red heartwood is a mixture.
- The author established the difference between the *in vivo* (red heartwood) and *in vitro* reaction products in the following: the product isolated from the red heart is a mixture of various oligomers containing more phenolic hydroxyl-groups and less quinon-groups, while in the products gained from the *in vitro* reactions under the given circumstances, there are more quinon- and less phenolic hydroxyl-groups.

4.5 From his research data the author concluded that the formation of the red heartwood chromophores is a result of many conjuncting factors, of which the pH of the tissue, quality and concentration of phenolic compounds and the quantity and activity of oxidoreductase enzymes are of major importance.
The diffusion of molecular oxygene beyond the „drying transition zone" together with the increased activity of the enzymes and the increased pH values of those tissues generates a chemical

environment, in which the formation of the red heartwood substances can take place.

4.6 The author has proven that red heartwood formation in beech is analogous to the *Juglans*-type obligatory coloured heartwood formation in many aspects.

- The concentration of phenolic compounds (flavan-3-ols) playing the major role in red heartwood formation show a monotonous accumulation from the bark towards the colour boundary.
- In front of the colour boundary, in the transition zone the *in situ* synthesis of phenolic compounds can not be detected, or takes place only in a slight degree.
- Accumulation and hydrolysis of succrose in the transition zone is mostly insignificant.

5. Possible utilisation of the research results

The exploration of the role of chemical substances, parameters and processes in the red heartwood formation of beech is a basic research, the data provided by the author contribute to the understanding of the chemistry of the physiological processes. Beyond that the results could:

- open up new possibilities in developing applicable non-destructive indication methods and in the chemical indication of red heartwood in living stems,
- give basis to develop new silvicultural methods in order to fight back red heartwood formation,
- help to implement technologies which can achieve colour homogenization, balance colour contrasts and enhance the colour stability of red heartwood.

6. Bibliography of personal publications considering the dissertation

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