STSM Scientific Report

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STSM information

Reference: Short-Term Scientific Mission, COST Action FP 1106

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Host: Prof.Dr. Katarina Čufar, Department of Wood Science and Technology, Biotechnical Faculty, University of Ljubljana, Slovenia; Katarina.Cufar@bf.uni-lj.si

STSM title: Micro-coring method to study wood formation in *Picea abies* Karst. and *Fagus sylvatica* L.

Place: Department of Wood Science and Technology, Biotechnical Faculty, University of Ljubljana, Slovenia

Reference code: COST-STSM-FP1106-11168

Period: 18/09/2012 – 14/12/2012

Introduction

In the period from September 18th till December 14th, 2012 during 13-week Short-Term Scientific Mission (STSM), I stayed at the Department of Wood Science and Technology of the Biotechnical Faculty in Ljubljana, Slovenia. I am a PhD student at the Department of Wood Technology at Faculty of Forestry in Zagreb, Croatia. I came to Ljubljana with intention to learn the methodology to study wood and phloem formation and the dynamics of cambial activity based on micro-core sampling in adult forest trees. My work was completed under the supervision of Prof. Dr. Katarina Čufar and in close co-operation with Dr. Peter Prislan.

This report provides a detailed overview of the working activities performed during the STSM and the scientific outcomes.

Purpose of the STSM

Seasonal dynamics of cambial activity and wood and phloem formation in trees can be followed by different methods, one of them is micro-coring. The aim of the STSM was twofold. First of all, I wanted to learn and conduct the complex procedure of micro-coring method. This required an introduction into practical application of the method itself through all phases, from micro-core sampling from trees, laboratory work to data analysis as a final step. However, in order to fully understand the process of wood formation, the study was conducted on a typical conifer Norway spruce (*Picea abies* Karst.) and typical hardwood European beech (*Fagus sylvatica* L.) from two sites in Slovenia. The

second aim was to acquire new knowledge that could be implemented in my research work, in relation to my PhD study, and application of the methodology acquired during STSM to home institution where it will be introduced.

It was expected that the results and acquired knowledge will contribute the COST STReSS Action as well, according to the main objectives: experience in collecting data using new methodology and thus organizing, integrating and exchanging knowledge with other participants.

Description of the work carried out

Research material and methods

At each of the two contrasting forest sites (Panška reka (PA) – 400 m a.s.l. and Menina planina (ME) – 1200 m a.s.l.), six beech and six spruce trees were selected for sampling. Because the growing and sampling period 2012 was already ending at the time of my arrival to Ljubljana, I visited the site and learn sampling at Panška reka on September 21, 2012. My work in laboratory was based on previously collected samples of micro-cores at weekly intervals during 2012 growing season, from March 23rd till June 18th (PA site) and from April 6th till June 15th (ME site). All samples were collected from stems of mature trees at approximately 1.3 m above ground.

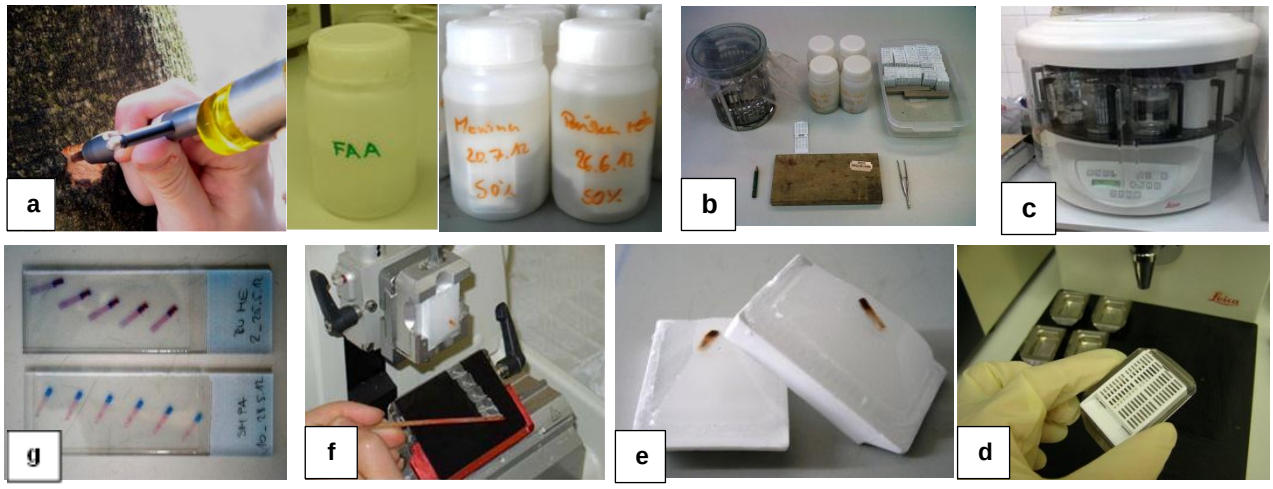

Figure 1 Practical micro-coring method application. (a) Field sampling of micro-cores, (b) Sample preparation, (c) Dehydration and paraffin infiltration , (d) Paraffin embedding, (e) Paraffin block trimming, (f) Cutting procedure, (g) Finished slides with cross-sections of spruce and beech.

The work performed during the STSM comprised the following tasks:

1. *Sampling of micro-cores*

   The initial step was to collect micro-cores containing phloem, cambial zone and outer xylem at weekly intervals using specialized Trephor tool (Rossi *et al.* 2006). Two micro-cores were collected from each tree at every sampling date. Micro-cores were immediately fixed in FEA (formalin-ethanol-acetic acid solution). After one week in FEA, they were displaced into 70% ethanol and permanently stored until the next task.

2. *Preparation of micro-cores for the embedding process*

   Micro-cores were oriented to mark the transverse side, shortened with a razor blade and put into a marked histosette. Marked histosettes were then put into a glass container with a tissue basket and 70% ethanol and placed in the tissue processor (LEICA TP1020).

3. *Dehydration and infiltration of micro-cores in the automatic tissue processor*

   The chamber, consisting of 12 reagent chambers (ethanol, Bio Clear and paraffin), was set to a suitable programme, according to which the 20-hour infiltration of samples was carried out.

4. *Micro-core embedding*

   This task included embedding of the micro-cores in paraffin, with special attention to the diagonal orientation of the sample in metal moulds. It was important that the sign on the histosette is visible and that, by adding paraffin, the upper surface is convex having in mind the additional shrinkage of hardened paraffin. After the cooling of paraffin blocks (for 15 minutes at room temperature and additional 15 minutes in the freezer), they were trimmed with knife. In case of destruction of the sample between xylem and phloem, another paraffin block had to be made.

5. *Cutting of the samples*

   Surface of the paraffin blocks was smoothened by cutting sections of 10 µm thickness until the surface of the micro-core was completely open. Before the next task, samples were moistened in water for 2-3 days.

6. *Cutting of the cross sections*

   Transverse cross-sections (thickness of 7 µm and 8 µm) were prepared with a Leica RM 2245 semi-automatic rotary microtome, using Feather N34H disposable low-profile steel blades. The sections were transferred to object slides covered with glycerol albumin for greater adhesion. Name of the sample was drawn on each slide and full slide holder with 10 slides was put for 20 minutes into the oven warmed to 70ºC.

7. *Preparation for staining and staining*

   For further staining, it was necessary to wash paraffin out of the slides with Bio Clear and 99% ethanol. Slide holder was put for 15 minutes in two consequent boxes of each reagent and then stained with mixture of safranin and astra blue for 20 minutes.

8. *Finishing the slides*

   After staining, the slides were washed in water and stored in 99% ethanol. From each slide uneven sections were removed, usable ones were embedded in Euparal and covered with cover slides. Air bubbles in mounting medium (if present) were removed and additional weight was put on each slide and left for at least two days, so that the resin hardened.

9. *Control of the cross sections*

   Observations were made using light microscopy (LM) - Nikon Eclipse E800 microscope to define whether any of the cross-sections is not satisfactory for interpretation and requires repetition of the cutting procedure.

10. *Data analysis*

    Cross-sections from the micro-cores were used to distinguish xylem cells of the growing tree-ring and to count the number of cells in the cambial zone, radial cell enlargement, secondary

wall thickening and lignification, the number of mature cells, tracheids in spruce or vessels and fibres in beech, and phloem growth ring. Light microscope (LM) was used to perform wood anatomical analyses, having in mind the sampling period that was analyzed. Together with Nikon Eclipse E800, DS-Fi1 digital camera and NIS Elements BR3 image analysis system were used.

The anatomical structure of spruce as a typical conifer is simpler than the structure of the hardwood beech. In spruce, the mentioned measurements and calculations on cambium and xylem growth ring were performed in three selected radial lines per sample. Data on the number of cambial cells in the cambial zone (CC1, CC2 and CC3); width of cambium (Cm1, Cm2, Cm3 in µm); number of cells in the phase of postcambial growth (PC1, PC2 and PC3); number of cells in the phase of secondary wall deposition and lignification (SW1, SW2 and SW3); number of mature tracheids (MT1, MT2 and MT3) and width of forming xylem growth ring (XY1, XY2 and XY3 in µm) were measured and entered in Excel worksheet. Due to more complex structure of beech wood and difficulty to count the cells of forming xylem growth ring in radial rows, the collected data included the number of cambial cells and width of cambium (in µm); width of part of forming ring with cells in the phase of postcambial growth (PC1m, PC2m and PC3m in µm); width of part of forming ring with cells in the phase of secondary wall deposition and lignification (SW1m, SW2m and SW3m in µm); width of the part of the forming ring with mature tracheids (MT1m, MT2m and MT3m in µm) and width of forming xylem growth ring.

Data on phloem growth ring, counted in three radial lines, included number of cells in early phloem (EP1, EP2 and EP3); number of cells in late phloem (LP1, LP2 and LP3); width of the early phloem (EP1m, EP2m and EP3m in µm) and width of the late phloem in the last formed phloem ring (LP1m, LP2m and LP3m in µm).

Description of the main results obtained and conclusion remarks

Method application and quality analysis

Preparation of cross-sections from micro-cores is a delicate operation, which demands considerable microtoming skill. Even then, the quality of very thin cross-sections in terms of uniform properties over their entire surface may not be completely achieved. Results presented in this report are based on experimental outcomes of micro-coring method. I used the method and cut approx. 350 slides (from 175 micro-cores). I made cross-sections of both wood species studied, from which 210 were of spruce and 140 were of beech. I focused on cutting and identification of potential causes of unadequate quality of cross-sections.

As already mentioned, the laboratory work has been done on micro-cores extracted in the period from the end of March until the middle of June, 2012. This period includes reactivation of cambium, onset of cell production and maximal production of cells. Our aim was to produce cross-sections of 7-8 µm. The micro-cores should be without deep cracks occurring on the outer surface.

During the process of removing micro-core from the cutting tube of the tool, compression of the unlignified tissues can occur thus increasing cell deformation in the meristems, collapse of enlarging tracheids or cracks in the thickening walls (Rossi *et al.* 2006). To obtain adequate quality, micro-cores should be collected 10 cm apart from each other, following a spiral up the stem to avoid wound effects (Prislan et al. 2011), and with a delicate pressure (generated with a needle or toothpick) separated from the cutting tube. In addition, micro-cores were extracted having in mind that reaction wood, if present, was avoided. Therefore, application of Trephor tool ensured high-quality samples in both softwood and hardwood species.

Micro-cores consist of parts with different consistency and density: a compact side composed of mature xylem and a soft side with phloem and differentiating cells. It was noted that they often break in half close to the cambial zone, leading to irreversible damage to the tissues and loss of material for observations. However, use of rotary microtome and paraffin embedding reduces problems related to small size and fragility of the samples.

Microscopic observation of quality of just prepared slides with cross-sections disclosed repetitive defects on cross-sections, most likely arising from application of cutting technique itself, as well as regarding to cambial activity dynamics during the period of growing season covered with sampling of micro-cores. Out of 350 I slides produced, around 25% were not satisfactory for further analysis. The percentage of inadequate sections was much greater in beech samples. From that number, approximately half were from the low elevation site of Panška reka and the other half were from the high elevation site of Menina planina. Despite the fact that similar type of defects were perceived with both wood species, the density of each wood seems to be one of the main causes of mechanical resistance to cutting. The density of spruce wood is 0,30...0,43...0,64 g/cm^3 (ρ_0), and of beech wood is 0,49...0,68...0,88 g/cm^3 (ρ_0) (Wagenfuhr, 2007.).

When comparing the cross-section defects in different trees at different sites, following conclusions could be derived. At Panška reka site, most defects on spruce cross-sections were noticed in two trees in the early phase of sampling, from the end of March, 2012 until the beginning of May, 2012. Regarding beech cross-sections, repeating defects in one tree were connected with sampling from the beginning of May, 2012 until the middle of June, 2012. At Menina planina site, critical period was during April, 2012 for spruce cross-sections in two trees. Regarding beech cross-sections, the ones sampled from the beginning of April, 2012 until the middle of May, 2012, mainly in three trees, were not satisfactory for interpretation.

Accoding to greater density of beech wood and differences in tissue hardness and consistency, it was harder to cut beech samples. However, the most common defect on cross-sections was cracking along the cambial zone. It was repeatedly noticed mainly in the early part of the season, with no regard to wood species. It could be assumed that wood samples extracted in spring or early summer are more likely to be damaged during the procedure because the cambium and enlarging cells have thin primary unlignified walls (Rossi *et al.* 2006). As well, at the beginning of the growth season, cell divisions occur faster than the differentiation, therefore the width of the cambium increases (Gričar 2007). Besides that, the cutting procedure demanding very thin cross-sections also affected the sensitivity of these cells.

Complex procedure of micro-coring method required every separated task to be performed according to directions (Internal manual). It was detected that some of the cross-sections were of very good quality, but some requested repetition of the cutting procedure, due to practical microtoming and procedural failure. Considering the performance of individual method task, possible remedies were outlined to avoid sectioning problems and to prevent unwanted outcomes. Firstly, it was quickly recognized that preparation of cross-sections can be done easier and more efficient if micro-cores are smaller (shorter). In addition, if smoothing the surface of the paraffin block does not completely open the micro-core, the water will not penetrate it evenly and thus facilitate the cutting. This would lead to small and narrow cross-section or even tissue distortion, with unsatisfactory structure for anatomical analysis. Furthermore, to achieve production of continuous paraffin strip during the cutting procedure, the paraffin block should have as regular square shape of the cutting surface as possible. The reason could also be found in blade bluntness. It usually results in each section coming off separately. Also, continuous movement of the hand wheel of the microtome in a steady rhythm is obligatory.

In the process of finishing the slides, enough Euparal should be added to assure that it spreads evenly under the cover slide. For this default, some of the slides were damaged during the procedure. One slide defect was evident even without the use of the microscope. On some slides, bubbles and stains spread around the cross sections. This appearance could be explained by the presence of water molecules which reacted with Euparal. After staining, slides are washed up in water and stored in highly concentrated ethanol (99%) until they undergo the last task of preparation. If ethanol is not regularly exchanged, it contains certain amount of water molecules which prevent the proper reaction of Euparal. Along with ethanol, all reagents should be exchanged after approximately 40 slides with cross-sections have been made, to maintain the quality of staining process.

According to rotary-microtome manual (Leica instructions manual), it was empirically concluded that the best cutting results were obtained when the clearance angle was around 3º. It was first considered that some damage was made to the cross-sections due to chosen clearance angle. However, varying the angle between 0º and 10º it did not appear to have a significant effect on absence of slip lines on cross-sections. In order to avoid any possible unforeseen variable which could affect the quality of the cross-sections, clearance angle of 3º proposed by the manual was adopted and applied to both wood species.

Turkulin (1996) concluded that the quality of the blade can influence the quality of the section to a much greater extent than the change in clearance angle. It is indisputable that cleavage of the material depends on the cutting geometry. It was quickly recognised that the condition of the blade and the microtome must be perfect for obtaining good cross-sections. The experience I gathered during this practical work confirmed that the condition of the blade is the most important parameter of the microtoming technique in order to obtain good and uniform cross-sections. The use of disposable blades is justified. I observed that the blade is blunt when it started to produce slip lines, or even damaged the whole cross-section. Since every successive cross-section of certain sample exhibited such error, it was obviously caused by the pressure and nonrecoverable deformation of the wood induced by improper condition of the blade. This could also be connected to production of thin sections. The routine adopted here was focused on the condition of the blade, assuming that even the slightest bluntness of the edge causes compression of the wood underneath it.

Moreover, compressed or folded cross-sections may result from insufficient thickness which should be increased, at least for 1 µm, or paraffin remains on the blade. The risks of increased distortion, compression and folding of the cross-sections could also be related to sample orientation towards the blade. Ideal orientation of the micro-core would be diagonal position in the paraffin block, xylem side facing upwards and phloem side facing downwards.

Data analysis

Mechanisms of cell production and maturation and dynamics of xylem formation have been widely studied in trees in order to better characterize stem radial growth. Fixation in FEA and LM enabled us to follow the productivity of the cambium in terms of number of newly formed cells, and of new xylem and phloem cells. In addition, it was possible to distinguish early and late phloem in the youngest non-collapsed phloem growth ring.

In cross-sections, we distinguished among the cambial cells (CC), differentiating xylem cells in postcambial growth (PC), secondary cell wall deposition and lignification (SW), mature cells (MT) and phloem growth ring (PH), as shown in Figure 2.

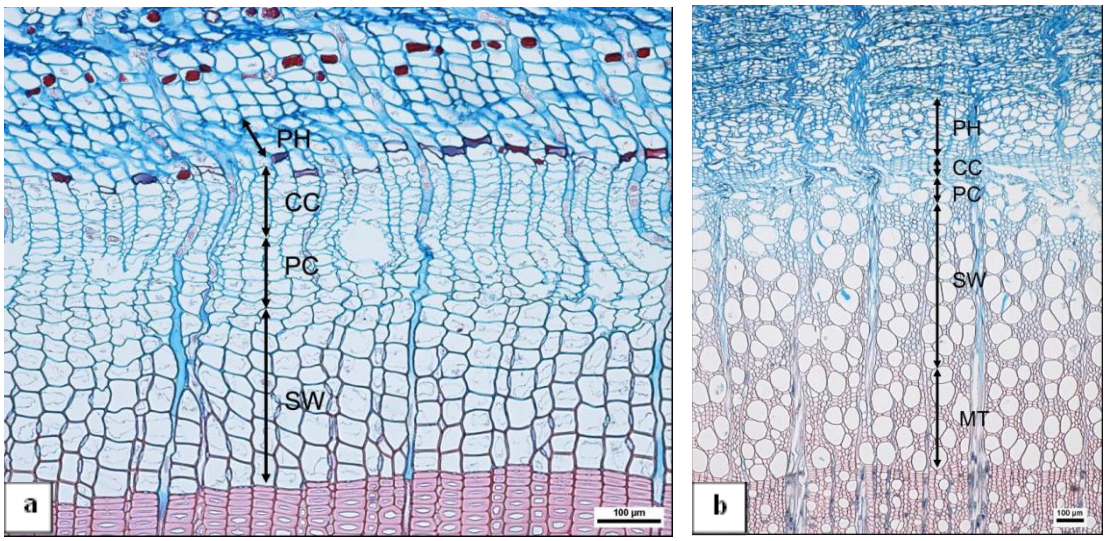

Figure 2 Light micrographs of transverse cross-sections of (a) spruce (Picea abies Karst.) and (b) beech (*Fagus sylvatica* L.) tissues from 2012 growing season, safranin and astra blue staining showing : cambial cells (CC), differentiating xylem cells in postcambial growth (PC), secondary cell wall deposition and lignification (SW), mature cells (MT) and phloem growth ring (PH).

The CC consisted of radially flattened cells with thin walls that stained blue with astra blue. The PC cells were larger and had thin, non-lignified, blue-stained primary cell walls. The deposition of secondary wall (SW) was observed under polarized light as the cell walls showed birefringence and their thickness started to increase. The beginning of cell wall lignification could be observed as the red staining by safranin gradually replaced the blue staining. When the process of differentiation was completed, the walls of mature cells (MT) were completely red stained and the cell lumina were empty (Čufar *et al.* 2008).

Since the main aim of my training was to learn and to qualify for application of micro-coring method, no detailed analysis, apart from measurements mentioned in the previous section of this report, was performed.

Even more close examination of cross-sections during microscopic data analysis helped me to recognize additional slides which were not satisfactory for interpretation. If the intended data measurements could not be performed, the procedure had to be repeated. Taking this into consideration, it was hard to find out exactly in which trees and site did the sampling and/or cross-section production give the most defects in cross-sections and what were the real causes for this. This could have been explained with the fact that no climatic data nor location of the trees at the particular site were taken into consideration.

After my work is completed, I can confirm the opinion that practical experience is a valuable learning tool. Implementation of the method into a specific research will enable me to gain additional experience and routine in its practical use.

Detailed study of new methodology as the one I performed during STSM opened up many new possibilities. My wish is to apply micro-coring method at my home institution. Since we do not have comparable laboratories and equipment, I am studying the possibilities of development of the methodology in home laboratories in co-operation with other institutions which partly have the needed equipment. I would like to start the research of wood anatomy and wood formation on domestic wood species, i.e. conifers (e.g. fir, spruce, pine) and hardwood species (e.g. oak, beech).

Future collaboration with the host institution

I consider this STSM a very successful one since both aims mentioned in the purpose section of this report were fulfilled and since I could start collaboration with the group of Prof. Dr. Katarina Čufar, in terms of sharing experiences and starting comparison of the data collected.

Projected publications to result from the STSM

Since the aim of the mission was to learn micro-coring method and to produce microscopic sections, and since I practiced the methodology on the material collected by the team in Ljubljana and I cut only a small portion (ca. 25%) of the collected material, my stay in Ljubljana did not enable me to produce publishable results. My intention is to publish an article on the micro-coring method and it's application and present it to both Croatian and international scientific audience. I intend to publish it in a scientific journal „Drvna industrija“ („Wood Industry“), which is included in the *Science Citation Index Expanded* database.

Acknowledgements

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References

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Confirmation by the host of successful execution of the STSM

A letter of confirmation of successful execution of the STSM (written by the host) is given on the next page.

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To Whom It May Concern

We confirm that Mrs. Iva Ištok from the Šumarski fakultet Zagreb visited the University of Ljubljana, Biotechnical Faculty, Department of Wood Science and Technology in the framework of STSM of the COST Action FP1106 and worked in the laboratories of the Chair for Wood Science for 3 months from 18 September until 14 December 2012 supervised by Prof. dr. Katarina Čufar and Dr. Peter Prislan.
The purpose of Iva's stay was to work on project with working title "Microcoring method to study wood formation". The candidate learned how to collect samples (micro-cores) in the field. She worked in laboratory and learned preparatory techniques including embedding, cutting, staining and preparation of microscopic slides. Finally she analysed the sections using light microscopy and image analysis systems, and we discussed possible ways of data evaluation. *Picea abies* and *Fagus sylvatica* mainly collected by the team in Ljubljana were used as model species for all steps of analyses
During her stay in Ljubljana the candidate studied relevant literature and discussed it during regular meetings with both supervisors. We also discussed possibilities to introduce the methodology in laboratories of the University of Zagreb and on possibilities that Iva starts doctoral dissertation in the field of wood formation. The application of the new method in Croatia and contribution of data would be of great interest of the Working group 1 of FP1106.

The training of Iva Ištok has been completed in accordance with suggested work plan.

Prof. Dr. Katarina Čufar
Head of the Chair of Wood Science

Prof. Dr. Miha Humar
Head of the Department of
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