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Methodical problems in studies on seasonal production of cambial xylem derivatives*

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Studies concerning the annual ring of wood formation in woody plants require repeated examination of the cambial region several times during the season. Anyone attempting this work with a microscope has to choose between one of the alternative methods allowing multiple collection of samples. The first consists in collecting stem samples (or stem segments) from different plants at viarious dates during the season, the second — in sampling repeatedly the stem of the same tree. Both methods have been employed by various authors (see literature reviewed by Grossenbacher, 1915; Brown, 1915; Ladefoged, 1952; Ermich, 1959). Irrespective of the method, great differences are regularly found in the radial cell number even between samples collected on the same day from healthy trees grown under comparable conditions. The differences comprise two kinds of variability — individual variability of trees, and the over-all stem variability due to the unequal manifestation of cambial activity at various points of the stem. Regardless of the physiological cause, the two kinds of variability result either from differences in the rate and duration (different dates of inception and cessation) of cambial xylem derivative production, or only from one of these variables. Collection of samples each time from different trees allows to investigate cambial activity in plants of any age at any point of the stem. The results are subject to the highest (individual) variability. The second method — collection of samples several times from the same specimen — eliminates the effect of individual variability, however, it can be applied only to trees of considerable stem thickness. In addition, the method may produce the side effect of wounding. Authors making the choice of the

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method of stem sampling for seasonal studies of wood differentiation in the past were seriously aware of the variability of the material and the effect of wounding. The problem has not been, however, satisfactorily studied, and discussed in the literature. This paper tries to fill partially this gap, at least for one kind of the commonly used plant material — adult trees of coniferous species.

METHOD

1. Collection and preparation of the material for microscopic examination

A number of trees with trunks about 100 cm in girth at breast height was selected in the large stand of 60-year-old pines in the Experimental Forests in Rogów. Dead outher bark was removed and samples of the inner bark, phloem, cambial region and the last yearly increment of wood were collected with a hammer-driven cylindrical punch 5 millimeters in diameter. Similar punches with smaller (Ermich, 1960, 1963) or greater diameters (Zahner, Oliver, 1962; Zahner, Lotan, Baughman, 1964; Whitmore, Zahner, 1966) have been used and found to be very convenient tool for stem sampling in studies concerning the differentiation of xylem. The side wall of the punch was deeply scooped out so the wood core could be removed from inside by pushing it backwards without destroying the cambial region. The cores were fixed in 70 per cent EtOH, hand-sectioned, stained with safranin and light green SF, and permanently mounted in Canada balsam. The counts of mature and differentiating tracheids were done under the microscope along four randomly chosen radial files on each transverse section. The distinction between dividing, growing, maturing and mature xylem was made according to the recommendation of Wilson *et al.* (1966).

2. Pertinent statistical formulas and notations

Introduction of a few notations may be useful for further studies concerning the choice of the best method of sample collection in the main experiment in which conclusions are drawn from analysis of differences between the average radial cell numbers found in a series of samples.

Notations used in the following section:

y_{ij} — radial cell number in the j-th sample taken from the i-th tree,
n — number of trees used in the main experiment,
s — difference between the numbers of the samples, (e.g. for two adjucent samples s = 1).

In the method searched for, the dispersion of the differences between averages due to the biological variability should be smallest. In other

words, the method is chosen which allows the smallest n to be sure at any required level of confidence (α) that the difference greater than the presupposed (allowable) one (M) is not a consequence of variability.

For testing some particular method of sample collection a preliminary experiment must be performed, where q samples are collected (according to the tested method) from p trees. Then: $j = 1, 2, ... q$; $i = 1, 2, ... p$; $s = 1, 2, ... q-1$.

If the compared averages are obtained from samples collected from different groups of trees n is calculated according to formula:

$$n = \frac{2\sigma^2 \cdot x_\alpha^2}{M^2} \tag{1}$$

where:

M — greatest insignificant difference between compared averages

x_α — value found from tables of normal distribution. It is equal to 1.96 for 95 per cent level of confidence, ($\alpha = 0.95$).

$$\sigma^2 = \frac{1}{(p-1)q}\left[\sum_{i=1}^{p}\left(\sum_{j=1}^{q} y_{ij}\right)^2 - \frac{1}{p}\left(\sum_{i=1}^{p}\sum_{j=1}^{q} y_{ij}\right)^2\right] \tag{2}$$

(σ^2 determined in preliminary experiment in which the method of sample collection from individual trees is not critical).

If the differences between samples of the same tree (calculated as averages from several trees) are to be analysed, the formula determining n for the main experiment is:

$$n = \frac{\sigma_s^2 \cdot x_\alpha^2}{M^2} \tag{3}$$

where:

M, and x_α — as in formula (1)

$$\sigma_s^2 = \frac{1}{p(q-s)-1}\left\{\sum_{i=1}^{p}\sum_{j=1}^{q-s}\left(y_{ij+s} - y_{ij}\right)^2 - \frac{1}{p(q-s)}\left[\sum_{i=1}^{p}\sum_{j=1}^{q-s}\left(y_{ij+s} - y_{ij}\right)\right]^2\right\} \tag{4}$$

[σ^2 determined in preliminary experiment where y_{ij+s} is the radial cell number in the (j+s)—th sample taken from the i—th tree].

It must be recognized, that the most important in this kind of experiments is the case when the difference of the average cell numbers between two adjacent samples is studied. Thus, the comparison of the tested method of sample collection should be mainly based upon n calculated at $s = 1$.

Some problems in the main experiment may require the direct analysis of the variability of the absolute radial cell numbers in samples collec-

ted from the same tree by the chosen method. The following standard deviation refers to this variability:

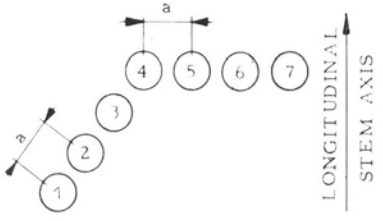
$$\sigma' = \sqrt{\frac{1}{p\,(q-1)}\left[\sum_{i=1}^{p}\sum_{j=1}^{q} y_{ij}^2 - \frac{1}{q}\sum_{i=1}^{p}\left(\sum_{j=1}^{q} y_{ij}\right)^2\right]} \qquad (5)$$

RESULTS AND DISCUSSION

1; Individual variability and the effect of the reciprocal situation of the samples

Seven samples from the stem of each of 12 trees were taken at breast height and the radial number of tracheids across the last fully differentiated annual ring was determined. Samples were 1 cm distant from each other and disposed along two lines as indicated in Fig. 1. Analysis

Fig. 1. Disposition of samples on the tree trunk.



of variance (Snedecor 1962) revealed a many times higher variability among trees than among, individual samples of the same tree (Table 1). When the variability between samples and discrepance are very small as compared with the variability among individual trees, a calculation according to formula (1) given in the previous section allows the following conclusion: in order to reach the level of 95 per cent probability that the difference of 1 cell between the mean radial numbers of tracheids obtained from two different groups of trees is not a consequence of individual variability (i.e. when $M = 1$), it would be necessary to sample about 5200 different trees in each group! This result rules out the possibility of investigation by this method of the seasonal course of xylem formation with a high degree of accuracy and certainty and implies the necessity of sampling repeatedly the same tree throughout the season.

Variability of differences between the cell numbers in samples taken along the horizontal line was similar to, or only slightly smaller than, the variability between samples collected along the oblique line (respecti-

Table 1

Variability in the number of tracheids across the annual ring of wood among twelve adult pine trees and among the seven individual samples taken from the trunk of each tree

Source of variation	Degrees of freedom	Sum of squares	Mean square
Trees	11	7548.3	686.2
Samples	6	227.4	37.9
Discrepance	66	1684.9	27.3
Total	83	9460.6	

ve variances: 21.5, 23.5). Using the formula (3), it can be calculated [for differences between the closest samples ($s = 1$) and for $M = 1$] that the respective n's in two methods are 83 and 90. Consequently, the first method which enables easier collection of samples was chosen for further investigation.

2. Circumferential variability

Samples in a series taken from the trunk on the same day at equal distances from each other and disposed along the horizontal line differ in respect to the radial number of tracheids which have been formed since the initiation of cambial xylem production at the beginning of the season. These differences represent the circumferential variability connected with an unequal rate, or also nonsimultaneous initiation and termination of cambial xylem production at various points of the woody stem (a particular case of over-all stem variability). The variability affects the differences between the radial number of cambial xylem derivatives found in samples collected at various time intervals during the season. The higher the variability and the more frequent the sampling the less accurate is the determination of the net gain in the radial cell number connected with current cambial production.

The effect of circumferential variability is greatly reduced if the experimenter uses average data obtained with samples taken from several trees instead of a single specimen. This method limits also the effect of the variability connected with a different manifestation of the circumferential variability between individual trees. The circumferential variability has been studied in two groups of twelve successive samples taken on the same day in winter at 1 or 2 cm distances along the circumferential line from trunks of 15 trees. Sampling at greater distances has been found impractical because only a small number of cores could be collected even from plants with considerable stem thickness. The mean radial number of tracheids in the two groups of samples and the variances referring to the variability of differences between the cell numbers of samples collected from the same tree are specified in Table 2.

Table 2

Least required tree number for investigation of differences between mean radial cell number in samples of wood collected from the tree trunk at 1 or 2 cm distance from each other

Distance between samples	Mean number of cells	Variance [1] σ_s	Size of sample population of trees [2]		
			0.5 [3]	1	2
1 cm	45.9	27.4	421	105	26
2 cm	44.0	64.1	985	246	62

[1]) Variance of differences between radial cell numbers in the neighboring samples (s = 1).
[2]) Least required tree number (n) for simultaneous investigation.
[3]) Greatest insignificant difference (M) at the 5 per cent level of risk.

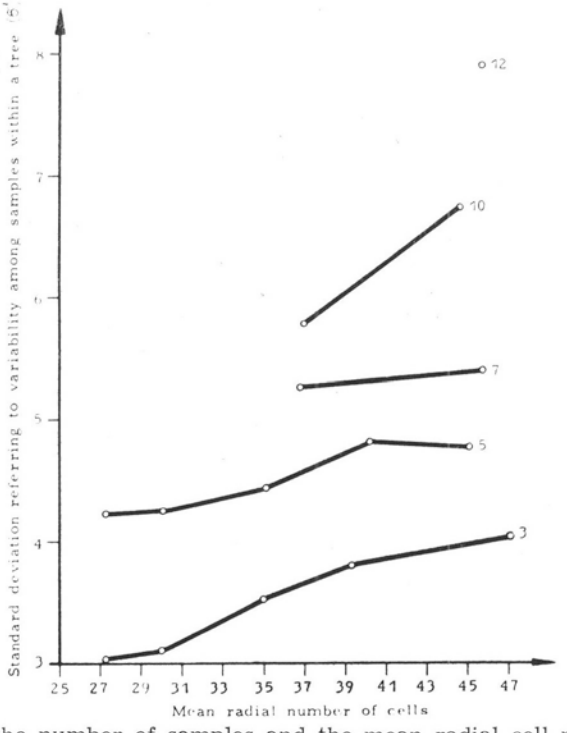


Fig. 2. Effect of the number of samples and the mean radial cell number upon the circumferential variability.

The least required number of trees which has to be sampled simultaneously to take a 95 per cent chance that the presupposed difference between the mean radial numbers of tracheids found in a series of samples collected from these trees will not be the consequence of circumferential variability, was calculated by the formula (3) given in the previous section.

The required number of trees for the experiment where the allowable *insignificant difference in radial cell number does not exceed 0.5, 1, 2,* tracheids is given in Table 2. The results point to the lower variability between samples taken at a 1 cm than at a 2 cm distance from each

other, and consequently, a smaller number of trees has to be sampled, if the former method is used.

The circumferential variability may be studied also by analysis of the radial cell number in the samples collected from the same tree [see formula (5)]. Then, it is seen, that its magnitude depends upon the mean radial cell number in these samples and upon the number of samples compared (Fig. 2). When the variability of differences between the radial cell numbers of these samples is studied, only the effect of the radial cell number is manifest and the effect of the number of samples is not observed. From Fig. 2 it may be concluded, that the circumferential variability increases as the season advances and reaches the maximum at its end. Consequently, these effects must be borne in mind when the production of xylem by determination of radial cell number at different points of the stem and various periods of the season is studied.

3. Effect of stem wounding

Mechanical disruption of stem tissues results in a severe disturbance of physiological processes which affects the activity of cambium and differentiation of its derivatives. The response of cambium extending over some distance laterally to the wound, recorded as a change in radial production of xylem elements and in the extensiveness of the two zones of tracheid differentiation, imposes a serious limitation on sampling repeatedly the stem of the same tree for studying activity throughout the season.

The preliminary experiment concerning this question involved samples taken along the circumferential line from stems of 23 trees, three times during the early summer and three times in autumn. Each sample was 1 cm distant from the previous one. Only one sample was taken from each tree on June 1. Three successive samples were taken on each of the five following dates specified in Table 3.

No significant influence of wounding associated with sample collection was noticed at 2-week intervals of sampling at any of the three distances (1, 2 or 3 cm) from the wound. The effect of wounding was found in samples collected 70 days after the previous term of sampling, however, it was significant only in the sample next to the wound.

The next experiment was designed to determine more precisely the time when the significantly detectable „wound effect" can be expected over some greater distances. Samples were collected along the circumferential line and at 1-cm distance from each other from 300 trees randomly assigned to 5 groups. The first time, six samples were taken from all trees on June 20 (to increase the wound effect). Only one sample of these (No. 0), the one to the extreme right, was afterwards used for determination of the radial cell number at the beginning of the experiment. Each

Table 3

Effect of wounding the trunk, associated with sample collection, upon rate of xylem production and radial diameter growth or maturation of tracheids. Averages from 23 trees of Pinus silvestris

Date of collection of sample	No. of sample	Radial number of cells				
		Cambial zone	Differentiating xylem		Mature xylem (tracheids)	Total
			Radially enlarging xylem	Maturing xylem		
		C	G	D	T	G+D+T
June 1	1	4.1	9.8	0.0 A *	0.0	9.8 A
	2	4.1	9.7	4.1 B	0.0	13.8 B
June 15	3	4.2	9.8	4.1 B	0.0	13.9 B
	4	4.2	9.9	3.9 B	0.0	13.8 B
	5	4.2	8.6	6.8 C	4.3	19.7 C
June 29	6	4.2	9.8	7.5 C	4.0	21.3 C
	7	4.2	9.9	6.6 C	3.7	20.2 C
	8	4.3	7.6	23.7 D	27.0	58.3 D
September 7	9	4.3	6.8	19.5 E	24.9	51.2 E
	10	4.3	5.4	18.6 E	25.3	49.3 E
	11	4.5	0.6	20.4 E	26.9	47.9 E
September 21	12	4.4	0.3	19.9 E	26.0	46.2 E
	13	4.4	0.4	10.3 E	26.2	46.9 E
	14	4.4	0.0	0.0	47.2	47.2 E
November 11	15	4.4	0.0	0.0	48.0	48.0 E
	16	4.6	0.0	0.0	49.0	49.0 E

* Different letters denote significant differences at 5 per cent level of risk.

group of trees was subsequently sampled only once more after a different period of time following the first sampling:

Group 1, on July 11 — after 21 days
„ 2, „ August 1 — „ 42 „
„ 3, „ August 22 — „ 63 „
„ 4, „ September 12 — „ 84 „
„ 5, „ November 14 — „ 147 „

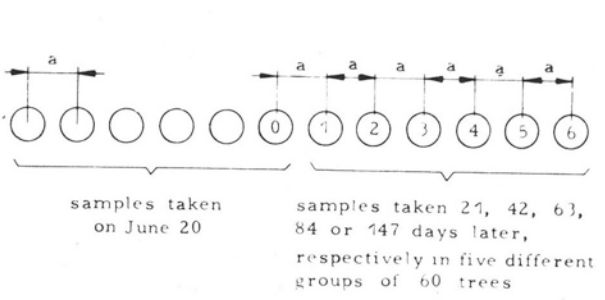


Fig. 3. Collection of samples from the stem for studying the effect of wounding.

Each time six samples (numbered 1 to 6) were taken from trunks of trees of the same group (Fig. 3). The first sample was taken at a 1-cm distance to the right of the last sample (No. 0) taken on June 20. Mean radial cell numbers in two zones of differentiating xylem and mature tracheids were determined in all samples (Table 4).

Table 4

Effect of wounding of the trunk, associated with sample collection, upon the rate of xylem formation in Pinus silvestris. Averages based on 60 trees, except the initial sample collected on June 20, which represents 300 trees

Date of sample collection	No. of sample *	Number of cells				
			Differentiating xylem			
		Cambial zone (C)	Radially enlarging xylem (G)	Maturing xylem (D)	Mature xylem (T)	G+D+T
June 20	0	4.36	6.11	5.65	8.84	20.60
July 11	1	4.42	5.93	6.30	14.25	26.48
	2	4.42	5.06	6.45	14.02	25.53
	3	4.45	5.48	6.85	14.42	26.75
	4	4.45	5.52	6.70	13.95	26.17
	5	4.52	5.36	6.85	13.27	25.48
	6	4.55	5.20	6.77	13.18	25.15
August 1	1	4.40	8.84	9.65	18.03	36.52
	2	4.37	6.87	8.75	17.30	32.92
	3	4.38	6.37	9.13	18.07	33.57
	4	4.28	6.30	8.57	17.40	32.27
	5	4.43	6.55	8.85	17.35	32.75
	6	4.43	6.23	8.75	17.07	32.05
August 22	1	4.60	7.24	18.33	26.55	52.12
	2	4.40	5.85	15.07	22.90	43.82
	3	4.37	5.28	14.58	23.52	43.38
	4	4.43	5.00	14.08	23.20	42.28
	5	4.45	4.65	14.90	23.15	42.70
	6	4.50	4.50	14.55	23.27	42.32
September 12	1	4.52	0.00	22.65	31.02	53.67
	2	4.45	0.00	16.94	28.48	45.42
	3	4.47	0.00	15.32	27.43	42.75
	4	4.50	0.00	15.15	27.70	42.85
	5	4.47	0.00	15.03	27.70	42.73
	6	4.50	0.00	15.65	27.62	43.27
November 14	1	4.47	0.00	0.00	53.13	53.13
	2	4.40	0.00	0.00	47.22	47.22
	3	4.40	0.00	0.00	44.35	44.35
	4	4.40	0.00	0.00	42.02	42.02
	5	4.33	0.00	0.00	42.90	42.90
	6	4.56	0.00	0.00	42.67	42.67

* Distance from the initial sample No. 0 in centimeters.

Table 5

The summary of analysis of differences *) between the radial cell numbers in samples taken horizontally at various distance from the wound on the stem of tree. Sample numbers denote the distance from the wound in centimeters. Average data from 60 trees. Capital letters — differences significant at 5 per cent level of risk; regular letters — insignificant

Period	Sample number							Period
		1	2	3	4	5	6	
June 20 — July 11	1		GdtP	GdtP	GdtP	GdtP	GdtP	1
	2	gdtp		gdtp	gdtp	gdtp	gdtp	2
	3	,,	gdtp		,,	,,	,,	3
	4	,,	,,	gdtp		,,	,,	4
	5	,,	,,	,,	dgtp		,,	5
	6	,,	,,	,,	,,	gdtp		6
June 20 — August 22	1		DtP	DtP	DtP	DtP	DtP	1
	2	GDtP		dtp	dtp	dtp	dtp	2
	3	,,	gdtp		,,	,,	,,	3
	4	,,	,,	gdtp		,,	,,	4
	5	,,	Gdtp	,,	gdtp		,,	5
	6	,,	,,	,,	,,	gdtp		6

June 20 — August 1 (Period)

June 20 — Sept. 12 (Period)

G, g — radially enlarging xylem; D, d — maturing xylem; T, t — mature xylem (tracheids); P, p — total production of cambial xylem derivatives

* According to Tukey's Q method in modification of Snedecor (1962).

The effect of wounding is first observed as the extension of the zone of enlarging xylem accompanied by an increase in the rate of new xylem elements production. No such effect was evident after 21 days following the first sampling. However, both effects were significant after 42 days, although only at a 1-cm distance from the wound (Table 5). The effect upon the zone of maturing xylem is not observed even 42 days after wounding. It becomes evident, however, after 63 days at a distance of

1 cm from the wound and at 2 cm after additional 21 days. The last observed is an effect of wounding upon the production of mature tracheids.

It can be concluded for the trees under study that collection of samples from the stem, at intervals up to 21 days at a 1-cm distance from each other, proves to be a safe method of studying xylem formation throughout the season. Up to these limits the method was found to be free of a measurable effect of wounding.

SUMMARY

Studies concerning formation of annual ring of xylem require often the comparison of radial numbers of tracheids obtained from a series of samples collected from the trunk of the tree at intervals during the season, because a high individual variability in the radial cell production makes it impractical to work with various trees each time a sample is taken. Studies basing upon multiple collection of samples from the trunk of the same tree are affected by the natural over-all stem variability of the radial cell number owing to the unequal manifestation of cambial activity at various points of the stem, and the effect of wounding. The effect of circumferential variability, a particular case of over-all stem variability, was found to be smaller when samples were taken at a 1-cm instead of 2-cm distance from each other. The magnitude of circumferential variability depends also upon the number of samples used for the analysis and on the mean radial number of cells in these samples. The effect of this variability is greatly reduced if samples are taken from several trees instead of a single specimen and averages are analysed. Samples collected at intervals up to 21 days with a hammer-driven cylindrical punch 5 mm in diameter proved to be free of the measurable effect of wounding at a 1-cm distance from the wound. This effect is evident after longer periods of time even at greater distances from the wound.

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Zagadnienia metodyczne dotyczące badań sezonowych nad tworzeniem się drewna

Streszczenie

Badania nad tworzeniem się rocznego słoja drewna często wymagają porównywania liczby cewek, jakie utworzyły się w kierunku promieniowym w pniu drzewa, z określonej serii próbek pobranych z pnia tego samego drzewa w różnym czasie w okresie sezonu wegetacyjnego. Konieczność pobierania próbek wielokrotnie z pnia tego samego drzewa, a nie każdorazowo z innego, związana jest z bardzo wysoką zmiennością indywidualną drzew pod względem produkcji komórek drewna w kierunku promieniowym, która uniemożliwia praktycznie posługiwanie się tą drugą metodą. Badania opierające się na wielokrotnym pobieraniu próbek z pnia tego samego drzewa obarczone są jednak ogólną zmiennością w obrębie samego pnia, związaną z niejednakowym przejawianiem się aktywności kambialnej w różnych punktach pnia. Wpływ zmienności obwodowej (szczególnego przypadku ogólnej zmienności w obrębie pnia) jest mniejszy, gdy próbki do badań pobierane są w odległości co 1 cm niż co 2 cm. Wielkość zmienności obwodowej zależy jednak także od liczby badanych próbek i średniej liczby komórek w tych próbkach. Wpływ tej zmienności jest znacznie ograniczony, gdy do badań pobiera się wielokrotnie próbki z pni większej liczby drzew zamiast pojedyńczego osobnika i gdy badania prowadzone są na średnich z tych kolejnych próbek. Próbki wybijane w okresach nie przekraczających 21 dni przy pomocy cylindrycznego dłuta o średnicy 5 mm nie powodują dającego się zauważyć wpływu zranienia na liczbę komórek w odległości 1 cm od miejsca pobrania próbki. Wpływ taki jest widoczny po upływie dłuższego czasu nawet w dalszej odległości od zranienia.