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Flavonoids from *Pinus sylvestris* needles and their variation in trees of different origin grown for nearly a century at the same area

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Abstract

Flavonoids in needles of Scots pine planted in 1912–1914 in Poland from seeds originating from different parts of Europe, were isolated, chemically characterised and analysed by HPLC. It was shown that flavonoid profiles were similar in all tested populations and were different from those previously reported for Scots pine seedlings. They included taxifolin, taxifolin 3′-*O*-glucoside, quercetin as well as quercetin 3-*O*-glucoside and 3′-*O*-glucoside. The quercetin 3-*O*-glucoside could be found only in a trace amount in all samples and quercetin 3′-*O*-glucoside appeared in all samples regardless their origin. The relative concentration of taxifolin 3′-*O*-glucoside, quercetin, taxifolin and total flavonoids showed dependence on the origin of seeds; needles from high latitude populations contained smaller amounts of these compounds. Presented data clearly indicate that Scots pine contain glycosidases specific for glycosylation at C-3′ rather than at C-3. Besides, they indicate that long lasting influence of similar environmental factors is not able to change genetic regulatory systems responsible for flavonoid biosynthesis.

Keywords: Pinus sylvestris; Flavonoids; Taxifolin; Quercetin glycosides; Geographic variation; Secondary metabolites

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1. Introduction

Scots pine (*Pinus silvestris* L.) is widely distributed and according to some reports 144 different "types" within this species can be recognised (Carlisle, 1960). The genus seems to be quite heterogeneous from morphological and physiological points of view. However, there are some distinct differences in secondary metabolite composition between different populations and also inside of a given population especially regarding phenolics.

As shown by previous studies of the flavonoid aglycones, in a given population of *P. sylvestris*, two chemomorphs coexist. One of them lacks taxifolin (T^-) and the other one contained *o*-dihydroxylated flavonoid quercetin and taxifolin (T^+) (Laracine-Pittet and Lebreton, 1988). The T^+ morphs could be found only at low elevations, while T^- were characteristic of high altitude or latitude populations. Qualitative differences in phenolic profiles and their concentration can be influenced by environmental factors (Nerg et al., 1994). They may play an important function in resistance to some pathogenic organisms (Lieutier et al., 1996) but some studies showed that taxifolin glucoside had no negative effect on insect performance (Larsson et al., 1992). Moreover, flavonoids can play an important function in protecting plants against UV-A and UV-B radiation and this protection is both concentration and structure specific (Sullivan and Teramura, 1992; Schnitzler et al., 1996, 1997; Robakowski and Laitat, 1999).

Herein we present a study where the flavonoid composition and concentration in the needles of Scots pine originating from five areas from Eastern Europe and Siberia and grown for over 80 years in Poland, have been analysed. The aim of this study was to establish whether a long lasting influence of the environment can modify flavonoid profiles and their total concentration.

2. Materials and methods

2.1. Description of the origin of trees and sampling

Trees were developed from seeds of Scots pine brought from diverse regions in Eastern Europe and Siberia (Table 1). The first experiment was established during 1912–1914 in the Ruda Forest belonging to the Institute of Soil Science and Plant Cultivation, Puławy, Poland (Oleksyn et al., 1993). The experimental area is situated at 51°37′N, 22°06′E, 140 m elevation and on a slightly south-east-facing slope. The site was managed by typical silviculture for 85 years (1912–1997). The other site was established in 1981 and is located near the Institute of Dendrology, Kórnik, Poland (52°15′N, 17°04′E, 70 m altitude). One-year-old needles were taken from trees of the same origin and from both sites (five trees per combination) in September 1997. They were freeze-dried and stored for analysis.

Table 1
Provenances of Scots pine seeds (*Pinus sulvestris* L.) used in the experiment

Sample	Region	Forest range	Coordinates[a]	
			Lat.N	Long.E
1	Kars	Songalugskoe & Borzomskoe	40°30′	42°00′
2	Wolyn	Luckoe	50°45′	25°18′
3	Pulawy	Las Rudzki	51°37′	22°06′
4	Jenisejsk	Jenisejskoe (Konsko-Jenisejskoe)	58°00′	62°00′
5	Arkhangelsk	Verkoleskoe & Lasskoe	63°50′	45°10′

[a] The data concerning geographic coordinates of individual ranges in which seeds were collected for the experiment are cited after Kurdiani (1932). Some coordinates are only approximate.

2.2. Preparative isolation of flavonoids

Powdered plant material (300 g) was defatted and depigmented in Soxhlet with CCl_4 and then extracted with methanol. After filtration the methanol was evaporated and the remaining solid was suspended in water. This was loaded onto a short column (3.5×7 cm) filled with RP18 phase (Baker). The column was first washed with water (discarded) and flavonoids were eluted with 40% methanol (Oleszek, 1988, Stochmal et al., 2001a,b,c). Removal of the solvent yielded solid (3 g), which was dissolved in water and loaded onto a column (3×40 cm; RP18, 40 μm, Merck). The column was eluted by a gradient of 0–100% methanol in water. Fractions (10-ml) were collected and analysed by TLC (DC-Fertigplatten Cellulose, Merck, 15% acetic acid). The fractions showing similar profiles were combined. Ten fractions containing two–three flavonoids were obtained. They were further separated/purified by CC (1.5×50 cm, RP18, 15–40 μm, Merck) using specific isocratic systems of acetonitrile–1% H_3PO_4 established from analytical HPLC separation. Five, pure compounds (**1–5**) were isolated.

2.3. Spectral analysis

^1H and ^{13}C NMR spectra were recorded at 399.952 and 100.557 MHz, respectively, in methanol-d_4 on a Varian Unity-400 spectrometer. The UV spectra were recorded with a Hewlett-Packard 8453 spectrophotometer.

2.4. Analytical extraction

For extraction, 1g of powdered material was refluxed for 1.5 h with 50 ml 70% methanol. The methanolic extract was evaporated and 1/5 of it suspended in water was passed through a RP18 SepPak cartridge (Waters Associates) preconditioned

with 5 ml of water. The cartridge was washed with 5 ml of water and flavonoids were eluted by 5 ml of 40% methanol. The flavonoid fraction was condensed to the final volume of 1 ml and used for HPLC determination.

2.5. High performance liquid chromatography (HPLC)

HPLC separation/determination was performed on Waters chromatograph equipped with 996 PAD detector, 616 Pump and Millenium software. Separation were performed on a RP18 column (4.6×250 mm, Eurospher 100, 10 μm, Säulentechnik) using linear gradient of 1% H_3PO_4–40% AcN in 1% H_3PO_4 delivered at a flow rate of 1 ml/min during 70 min. Detection and calibration were performed at 350 nm. Three independent runs were performed for each extract, and particular flavonoids were identified by comparing retention times and UV spectra with those of isolated and identified standards. Quantification was based on external standardisation by employing calibration curves in the range of 0.1–1.0 mg/ml of reference compound.

2.6. Statistical analysis

For all variables statistical differences between the provenances were calculated by analysis of variance. The relationship between the latitude of the seed origin and the flavonoids were examined by regression analyses, and we do not assume that direct casual relationship are involved. A Ward's hierarchical clustering method was used to compute cluster groups of Scots pine populations based on flavonoids content at two sites (interaction between sites and populations were not statistically significant; $p \geq 0.14$, see Table 4). All statistical analyses were done with JNP software (version 3.1.6.2, SAS Institute, Cary, NC, USA).

3. Results and discussion

High performance liquid chromatography of flavonoid fraction from the needles of Scots pine revealed the presence of about ten compounds (Fig. 1). Only five of them based on their absorption spectra (Photodiode Array Detection) were classified into the flavonoid group (Fig. 2). These five compounds were successfully separated on C18 column and their structures fully established by spectral techniques (^{1}H, ^{1}H-COSY, NOE and ^{13}C NMR). Compounds **1** and **2** showed UV absorption maxima at 290 and 327 (sh) nm identical to those reported for taxifolin (Mabry et al., 1970). The ^{1}H and ^{13}C NMR data of **2** are in agreement with that reported for dihydroquercetin (taxifolin) (Agrawal et al., 1980; Shen and Theander, 1985). Compound **1** had six extra signals in its ^{13}C spectrum, characteristic of glucose. The glucose position was assigned at C-3′ after the comparison with the ^{13}C NMR data of the B ring of taxifolin (**2**) and compound **1**. A downfield shift of the *ortho*-carbons, C-2′ ($\Delta\delta_{1-2}$=2 ppm) and C-4′ ($\Delta\delta_{1-2}$=2 ppm) and *para*-carbon C-6′ ($\Delta\delta_{1-2}$=3.7 ppm) are in agreement with glucosylation at C-3′ (Agrawal, 1989). The UV-NMR spectroscopic

Table 4
Concentration of flavonoids[a] in needles (mg/g dry matter) of Scots pine of different origin grown in two sites in Poland of five trees (two samples per tree were used) of each population. (Standard deviations for the measurements are given in parentheses)

Site	Provenance	**1**	**2**	**3**	**4**	**5**	Total
Pulawy	Kars	0.90 (0.03)	0.48 (0.07)	0.000 (0.000)	0.80 (0.32)	0.68 (0.51)	2.85 (0.89)
	Wolyn	0.97 (0.43)	0.62 (0.26)	0.002 (0.001)	0.75 (0.47)	0.35 (0.13)	2.71 (1.26)
	Pulawy	0.76 (0.15)	0.58 (0.21)	0.002 (0.000)	0.92 (0.18)	0.51 (0.01)	2.76 (0.23)
	Jenisejsk	0.59 (0.31)	0.34 (0.26)	0.017 (0.020)	1.00 (0.02)	0.23 (0.32)	2.16 (0.91)
	Archangelsk	0.07 (0.02)	0.02 (0.03)	0.002 (0.000)	0.59 (0.06)	0.00 (0.00)	0.68(0.11)
	Mean	0.66	0.41	0.005	0.81	0.35	2.23
Kornik	Kars	1.52 (0.00)	0.29 (0.02)	0.015 (0.008)	1.76 (0.36)	0.17 (0.00)	3.76 (0.32)
	Wolyn	1.62 (0.33)	0.26 (0.03)	0.003 (0.001)	1.32 (0.07)	0.17 (0.04)	3.38 (0.40)
	Pulawy	1.43 (0.51)	0.27 (0.07)	0.007 (0.004)	1.10 (0.01)	0.38 (0.08)	3.18 (0.47)
	Jenisejsk	0.83 (0.33)	0.25 (0.04)	0.003 (0.001)	1.18 (0.57)	0.29 (0.12)	2.56 (0.96)
	Archangelsk	0.04 (0.01)	0.01 (0.00)	0.002 (0.000)	1.10 (0.37)	0.00 (0.00)	1.16 (0.36)
	Mean	1.09	0.22	0.006	1.29	0.20	2.81
Mean		0.88	0.32	0.006	1.05	0.28	2.52
$P>F$[b]							
Site		*0.006*	*0.012*	*0.683*	*0.006*	*0.120*	*0.094*
Prov		*0.001*	*0.009*	*0.475*	*0.444*	*0.064*	*0.004*
Site×Prov		*0.356*	*0.407*	*0.139*	*0.411*	*0.364*	*0.982*

[a] **1**, taxifolin 3′-*O*-glucoside; **2**, taxifolin; **3**, quercetin 3-*O*-glucoside; **4**, quercetin 3′-*O*-glucoside; **5**, quercetin.
[b] Statistical analysis (ANOVA effects $P>F$) were conducted using software (JMP version 3.05, SAS Institute, Cary, N.C.).

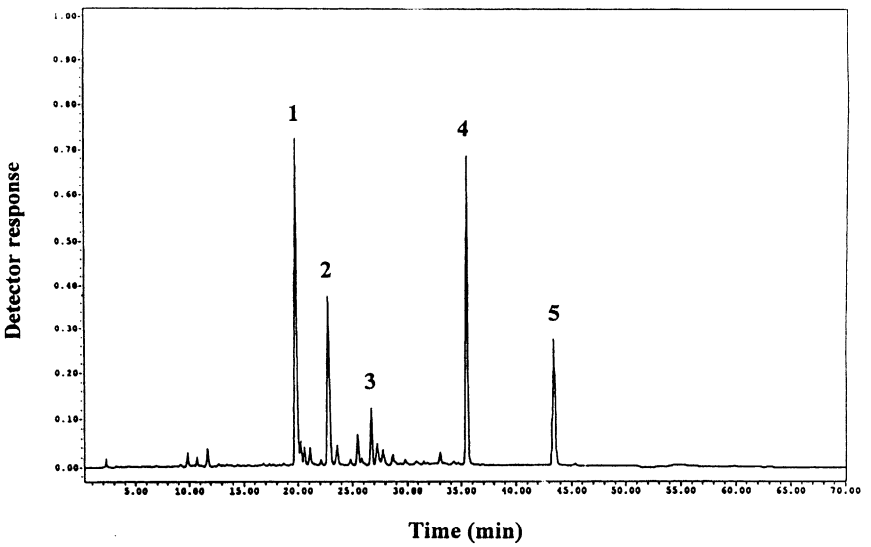

Fig. 1. HPLC profile of flavonoids from Scots pine needles (320 nm). **1**, taxifolin 3′-*O*-glucoside; **2**, taxifolin; **3**, quercetin 3-*O*-glucoside; **4**, quercetin 3′-*O*-glucoside; **5**, quercetin.

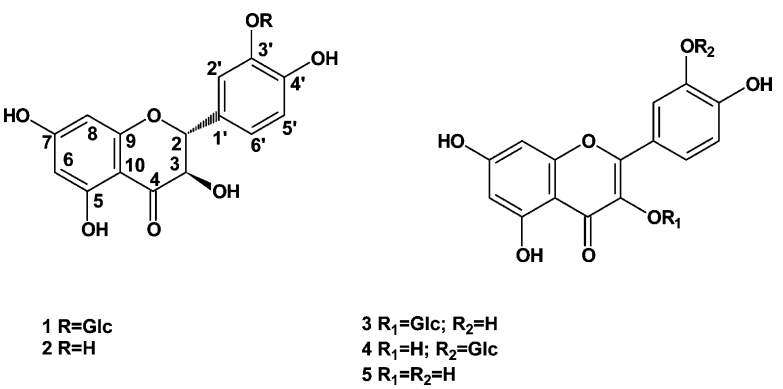

Fig. 2. Flavonoids identified in the Scots pine needles: **1**, taxifolin 3′-*O*-glucoside; **2**, taxifolin; **3**, quercetin 3-*O*-glucoside; **4**, quercetin 3′-*O*-glucoside; **5**, quercetin.

data of **1** (Tables 2 and 3) are in good agreement with the values reported for 3′-*O*-glucopyranoside taxifolin (Shen and Theander, 1985).

Compounds **3**, **4** and **5** showed UV absorption maxima at 257 and 352, 252 and 364, 255 and 370, respectively, which were characteristic for quercetin derivatives. The retention time and PAD spectrum for compound **5** were identical to those of quercetin standard, confirmed by the examination of ^{1}H and ^{13}C NMR data (Wenkert and Gottlieb, 1977; Chung et al., 1999). Compounds **3** and **4** showed in their ^{13}C spectrum besides quercetin signals, six additional peaks attributable for glucose moiety. Compound **3** on HPLC showed retention time and spectroscopic data identical

Table 2
^{13}C NMR data of flavonoids **1–4**

C	**1**	**2**	**3**	**4**
2	84.6	85.0	158.4	147.3
3	73.3	73.6	135.6	136.5
4	198.1	198.3	179.4	177.4
5	165.2	165.2	163.0	162.6
6	97.3	97.3	99.8	99.3
7	168.4	168.6	165.9	165.5
8	96.2	96.3	94.7	94.6
9	164.4	164.4	158.9	158.9
10	101.9	101.8	105.6	104.5
1′	129.8	129.8	123.0	124.4
2′	118.0	116.0[a]	116.0	118.1
3′	146.3	146.2	145.9	146.7
4′	149.1	147.1	149.8	151.6
5′	116.9	115.8[a]	117.5	117.1
6′	124.6	120.9	123.2	125.1
1″	103.7		104.3	104.4
2″	74.7		75.7	74.8
3″	77.4		78.1	77.6
4″	71.3		71.2	71.2
5″	78.0		78.3	78.4
6″	62.4		62.5	62.4

[a] May be interchanged.

Table 3
^{1}H NMR data of compounds **1-4**

H	**1**	**2**	**3**	**4**
2	4.96 d *J*=12 Hz	4.90 d *J*=11 Hz		
3	4.56 d *J*=12 Hz	4.50 d *J*=11 Hz		
6	5.90 sa[a]	5.91 d *J*=2 Hz[a]	6.37 d *J*=1 Hz[a]	6.44 d *J*=2 Hz[a]
8	5.87 sa[a]	5.87 d *J*=2Hz[a]	6.19 d *J*=1 Hz[a]	6.19 d *J*=2 Hz[a]
2′	7.37 d *J*=1 Hz	6.96 d *J*=2 Hz	7.70 d *J*=2 Hz	8.12 d *J*=2 Hz
5′	6.89 d *J*=8 Hz	6.80 d *J*=8 Hz	6.85 d *J*=8 Hz	6.97 d *J*=8 Hz
6′	7.08 dd *J*=8 Hz	6.84 dd *J*=8 and 2 Hz	7.57 dd *J*=8 and 2 Hz	7.86 dd *J*=8 and 2 Hz
1″	4.84 d *J*=7 Hz		5.24 d *J*=7 Hz	4.86 d *J*=7 Hz

[a] May be interchanged within the same column

to those of quercetin 3-*O*-glucopyranoside (isoquercetrin) (Wenkert and Gottlieb, 1977; Oleszek et al., 1988). Compound **4**, had higher retention time than flavonoid **3** and downfield shifts for signals of C-2′, C-4′, and C-6′ in its ^{13}C NMR spectrum suggested a glucosylation in C-3′ as observed in compound **1**. The NOE effect

observed in H-2′ signal when the anomeric proton signal (H-1″) was irradiated, definitively confirmed the structure of compound **4** as quercetin 3′-*O*-glucopyranoside. As far as we know this is the first report of ^{1}H and ^{13}C NMR values for quercetin 3′-*O*-glucopyranoside.

Fig. 1 presents a representative HPLC profile of the phenolics present in the needles of 85-year-old trees. Thus, this profile differs distinctly form the one reported for 63-day-old Scots pine seedlings grown under ambient UV radiation (Turunen et al., 1999). These seedlings contained predominantly quercetin and kaempferol glycosides together with their derivatives diacylated with coumaric acid. Also Schnitzler and co-workers (1996, 1997) reported similar flavonol composition when studying the influence of enhanced UV radiation on the composition, concentration and localisation of flavonols in Scots pine seedlings. It was reported that UV-B irradiation resulted in an increase in the concentration of diacetyl derivatives and their deposition in the epidermis, while the level of non-acylated flavonoids remained unchanged, suggesting the protective function of acylated compounds. These results were not confirmed in the experiments of Turunen and co-workers (1999), who reported a decrease in the concentration of non-acylated flavonols with decreasing UV-B and UV-A irradiances, while the quantity of diacylated flavonol glycosides was virtually unaffected by 58-day UV enclosure experiment. Our experiments with 2-year-old pine seedlings (unpublished data) showed the presence of four quercetin (including 3-*O*- and 3′-*O*-glucosides) and two kaempferol glycosides with no traces of acylated forms or free aglycones.

Thus, it seems that the flavonoid composition of the Scots pine is strongly dependent on the age of the plants used for analyses. The vast majority of experiments performed with Scots pine were done with one-year-old or younger seedlings, while a few studies were undertaken on adult trees. As pointed by Sullivan and Teramura (1992) there is a need for multiple season research on the effects of the increased UV-B on forest trees, because of their economic importance and their global distribution. Flavonoids are recognised as one of the major principles responsible for protection of needles from UV-B and UV-A radiation, but the mechanisms of such a protection can be totally different in seedlings and in old trees. Seedlings may be protected by the synthesis of acylated forms of flavonoids which, as shown by Schnitzler and co-workers (1996), comprised 79% of total absorbance at 300 nm. Acylation may enhance the absorbing capacity of flavonoids, e.g. acylation of apigenin and luteolin glycosides with coumaric, ferulic or synapic acids resulted in significant increase in specific absorption of band I (300–330 nm) (Stochmal et al., 2001a, b, c). So far nothing is known about the effectiveness of the UV-protection mechanisms of forest trees. However, as shown in the present research, needles from trees do not contain acylated flavonoids but instead they do contain taxifolin and its 3′-*O*-glucoside with the maximum absorption at 290 nm. These two compounds may give effective protection against UV radiation. Also the presence of quercetin might be crucial for UV protection. Both quercetin and taxifolin, as free aglycones, are the best antioxidants and free radical scavengers for a number of different classes of flavonoids tested (Burda and Oleszek, 2001). Thus, scavenging by free aglycones of highly reactive radicals may protect the plant from nucleic acid (i.e., DNA) and photosynthetic

apparatus damage. More research, however, is needed on the localisation, absorbing and scavenging effectiveness of quercetin and taxifolin derivatives in needles originating from old trees, similar to that as was done with seedlings (Schnitzler et al., 1996, 1997).

The relative amounts of the five compounds considered differed according to the original provenance of the trees (Table 4). The 3-*O*-glucoside of quercetin could be found only in trace amounts in all samples tested. By contrast both quercetin and taxifolin glucosides substituted at C-3′ were dominant compounds, while their aglycones could be also found. The ratio of the 3′-*O*-glucosides and of their respective aglycones (taxifolin and quercetin) was far higher in the profiles of the trees grown in the Kornik station than in the Pulawy forest and that was true for almost all-original provenances. The reason for such differences between sites does not seem to be the effect of the site climatic conditions, but rather was presumably attributable to the age of plantations; the Kornik plantation was established in 1981, while Pulawy trees have been grown since 1912–1914. This may indicate that the needles of the Scots pine contain glucosidases specific for glucosylation at C-3′ rather then C-3 and this feature was characteristic for all samples regardless their origin. It may suggest that this kind of glucosylation may be Scots pine specific and may serve as a marker in the phylogeny of conifers, similar to other flavonoid structural enzymes used for studying the evolution of phenolics in plants (Stafford, 2000).

Some differences between the concentrations of particular compounds were also found in samples from the trees of different origin. The origin of seeds had the most significant influence on the level of flavonoids in needles (Table 4). This relationship was evident both for total flavonoids as well as for the three of the five identified compounds—taxifolin, quercetin and taxifolin 3′-*O*-glucoside. Interestingly, all these three compounds seem to be crucial for UV defence, as discussed above. The site effect was minimal and for total flavonoid content was marginally significant (p=0.1) and there was no significant interaction found both for total flavonoid content and for particular compounds. This indicates that, regarding flavonoid concentration, both Kornik and Pulawy populations reacted in a similar way. Thus, presented data (Table 4) indicate that the level of flavonoids (excluding quercetin 3-*O*- and 3′-*O*-glucosides) is genetically controlled and is specific for populations of each different origin. The results presented in Fig. 3 indicate that neighbouring populations from Pulawy and Wolyn were closely related based on flavonoid concentration; the most distinct from the others was the most northern population originating from Archangelsk.

The relationship between the flavonoid level and the latitude of the population origin was very evident, which is exemplified in Fig. 4 and in Fig. 3 for total flavonoid concentration. This finding was supported by high values of r^2 coefficients, which indicated that in 98% for Pulawy and in 99% for Kornik, the concentration of flavonoids in needles of different Scots pine populations depended on the latitude of the population origin. This can be explained by the protecting function of flavonoids against UV radiation. At higher latitudes, the lower light intensity does not force the plants to produce metabolically costly protective flavonoids (Pare, 2000). This remains in general agreement with findings published by Nerg and co-workers (1994)

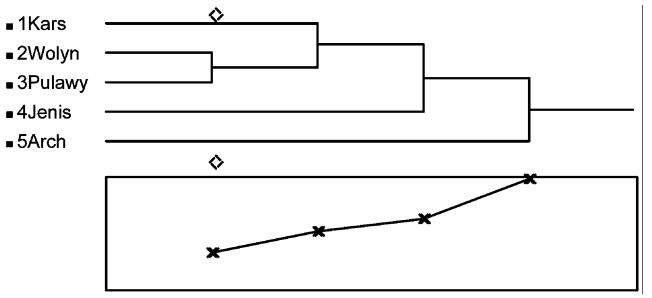

Fig. 3. Dendrogram of cluster groupings of provenances of Scots pine based on similarities of five flavonoid content. The plot beneath the dendrogram presents points for each cluster. The distance and curvature between the points represents the distance between clusters.

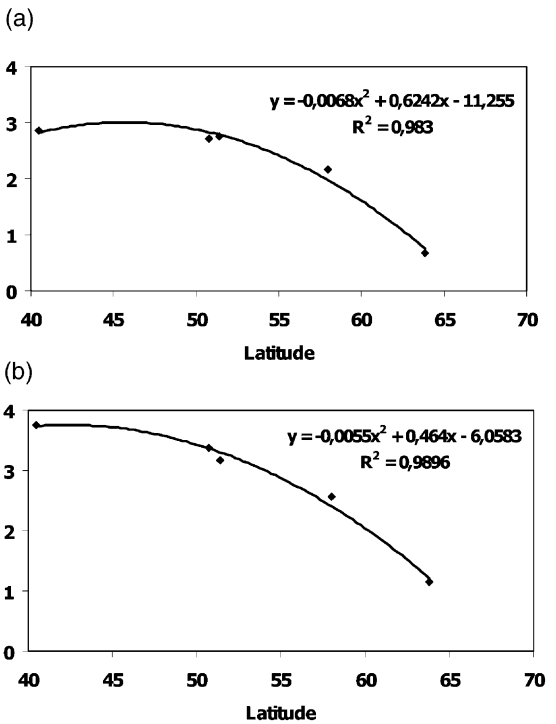

Fig. 4. Differences in total concentration of flavonoids in trees of different origin grown in two sites: A, Pulawy; B, Kornik.

showing that in the most northern location, the concentration of total monoterpens and total phenolics were lower. However, our findings remain in disagreement with the following statement from these authors: “results suggested that origin of seed material is not so important in affecting concentration of secondary compounds as are environmental factors”. In the present research the evident decrease in total fla-

vonoids as well as in some individual compounds suggests that the origin of seeds was an important factor determining the concentration of these compounds. Moreover, the data presented indicate that 88-years of the influence of environmental factors did not change the genetic regulatory system, developed over centuries, responsible for flavonoid biosynthesis. This is a quite astonishing finding and needs to be further supported by the research on other secondary metabolites using better representation of Scots pine populations.

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