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Studies on influence of rapeseed vegetation stages on level of phenolic compounds

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Abstract

A study was conducted to evaluate the changes in native antioxidants in rapeseed (*Brassica napus* L), which take place during crop growth. During the growth, in anatomical parts of plants (Kana and Spencer varieties), the increased content of hydrophilic antioxidants (phenolic compounds) was observed. During the plant maturation the decrease of phenolic acids was observed both in leaves during blooming and in hulls. During that time, total phenolic acid content decreased by 50% in Kana and by 40% in Spencer. The profile of phenolic acids in sampled rapeseed varieties was characterized by the highest level of sinapic acid and its low molecular derivative. It was proved, that during successive stages of maturation, the content of phenolic acids varied in anatomical parts of the plant. This may suggest different biological functions of particular phenolic compounds.

***Key words**: rapeseed, phenolic compounds, SPE, HPLC*

Introduction

In Poland, rapeseed is a cultivated oil crop of great economic significance. It is widely used in fat and food, pharmaceutical, chemical and fuel industries. In plants, phenolic compounds play various roles. They can be substrates in biosynthesis (for example, caffeic acid is a precursor to lignins). They are generated in order to protect plants against harmful action of ultraviolet radiation. Mutants of the *Arabidopsis* plant, incapable of synthesizing the phenolic compounds, were more susceptible to destructive action of ultraviolet rays (Bieza and Lois, 2001). Phenolic acids such as ferulic and p-cumaric acid occur as substances filling lignins with which they are ester-connected. In this case they act as inhibitors of cellulases produced by pathogenes hindering their penetration through the membrane inside the cell (Haddock *et al.*, 1982, Dixon and Paiva, 1995, Matsuki, 1996, Solecka *et al.*, 1999, Booij-James *et al.*, 2000, Shetty and McCue, 2003). Phenolic compounds such as red or blue anthocyanins, or yellow aurones and chalcones, attract pollinating insects. *In vitro*, majority of phenolic compounds display considerable antioxidant and anti-radical activity. Plant phenols stimulate oxidative processes while reducing metals just like vitamin C (Sugihara *et al.*, 1999). Apart from this, in the presence of nitric oxide (NO) some flavonoids display pro-oxidative activity (Ohshima *et al.*, 1998). Antioxidant activity of plant phenols is affected by the location and number of hydroxyl groups, polarity, and solubility and also stability of phenolic compounds during the treatments carried out (Decker, 1998). Energy of binding hydrogen with oxygen in a hydroxyl group connected with the aromatic system is much smaller than in aliphatic compounds. This causes considerable hydrogen mobility and conversion of phenolic compounds into phenoxyl radicals, and then, as a result of their linking, into quinones' derivatives (Burdyn and Nebesny, 2005). Introduction of additional hydroxyl groups in the aromatic ring increases the antioxidant activity. This results from stabilisation of phenoxyl radicals through formation of a hydrogen bond between these groups. Hence greater activity of the caffeic acid when compared to the coumaric one, substituting hydrogen in a hydroxyl group with a methyl group such as ferulic acid affects antioxidant properties in various ways depending on the polarity of the environment (Rice-Evans *et al.*, 1996).

Phenolic compounds occur universally in human diet, and most of them have antioxidant properties.

Biosynthesis and gathering of these phenolic compounds in plants are diverse processes monitored endogenously during plant growth. Also, they can be controlled exogenously by factors such as light, temperature and damages. Phenylalanine, formed on the shikimic acid path is a precursor of majority of phenolic compounds in higher plants (Saunders and Olechno, 1988, Schmidt and Schneider, 1999, Saltveit, 2004). Similarly, hydroxycinnamone acid, and particularly its enzyme with Co-A, is a common element in phenolic compounds such as cinnamone esters and amides, lignins, flavonoids and condensed tannins (Whetten and Sederoff, 1995, Kajita *et al.,* 1997). Phenylalanine / hydroxycinnamone path is the main metabolic path, the so-called phenylpropyl path in which deamination of phenylalanine occurs under the influence of phenylalanine ammonolysis directly to the hydroxycinnamone acid and its active forms (Hoffmann *et al.,* 2004).

Modern methods of seed composition modeling aim not only at obtaining varieties resistant to vermin, pathogens and abiotic agents, but also at increasing the content of valuable bioactive substances occurring in rape. Studies on biosynthesis of native lipophilic and hydrophilic antioxidants in the rape seed are in progress. They require not only experiments at the molecular level, but first of all verification of the introduced modifications in the field culture conditions.

Materials and Methods

Biosynthesis of native antioxidants (phenolic compounds) was investigated in two population varieties of the "00" rapeseed: Kana and Spencer. Kana, registered in 1997, is an early variety with increased oil content. Spencer *Nll*, registered in 2002, has lower level of the linoleic acid. Samples were drawn from the Przybroda experimental fields of the PoznaD University of Life Sciences in 2003. During crop growth, samples were collected at specific stages of the plant development: leaves at the time of flower formation and the flower buds (phase 55), leaves at the time of blooming and flowers (phase 65), leaves at the time of hull formation and the hull (phase 71) and seed (phase 85).

Preparation of samples for determination of phenolic compounds

After each harvest, green parts of plants were deep-frozen and then lyophilized using the Hetosicc Freeze Dryer Fd3 lyophilizer. The prepared samples were stored in tightly closed glass jars, under nitrogen, with no access of light. The rape seeds had been thoroughly ground in the laboratory and then subjected to defatting with hexane by multiple extraction of fat in the Soxhlet apparatus (Extraction System B-811, Büchi Labortechnik AG, Flawil, Switzerland).

Preparation of extracts of the phenolic compounds

Phenolic compounds in the leaf or seed samples were extracted three times with 80% water solution of methanol 1:3 (v/v); each time the samples were being shaken for 30 min. The obtained extracts were mixed and the solvent was evaporated using a rotary vacuum evaporator (Rotovapor-El, Büchi Labortechnik AG, Flawil, Switzerland). The remnants were quantitatively transferred with the help of 80% water methanol solution to 25 cm^3 measuring flasks.

Determination of total polyphenols

The content of phenolic compounds in methanol extracts was determined according to the colorimetric method with application of the Folin-Ciocalteu reagent (Sigma-Aldrich, St.Louis, MO, USA). 5 cm^3 distilled water and 0.5 cm^3 Folin-Ciocalteu reagents were placed in a measuring flask of 10 cm^3. Then 0.2 cm^3 of the examined extract was added and the whole content of the flask was thoroughly mixed. The prepared solution was left for 3 min. at room temperature; then 1 cm^3 of the saturated Na_2CO_3 solution was added and the flask was filled up with distilled water up to the mark. Absorbance was measured at λ_{max} 725 nm exactly 1 h from the time the sample had been prepared. On the basis of the standardization curve, the total content of phenolic compounds in the rapeseed extracts was determined and expressed as the sinapic acid (Grigordiadou *et al.,* 2005).

Isolation of phenolic compounds from the plant's green parts and seed

In order to isolate fraction of phenolic acids, the Chromabond® System (Macherey – Nagle, Germany) was applied together with the SPE Bakerbond spe™ columns filled with a quaternary amine (500 mg). The process comprised of 4 stages: I° conditioning of the columns (10 cm³ methanol, 10 cm³ distilled water and 10 cm³ 0.15% solution $NaHCO_3$); II° placing of the sample (5 cm³); III° washing of the column (15 cm³ 0.15% solution $NaHCO_3$); IV° WKF elution with a mixture of 0.2 M H_3PO_4 and methanol (2:1 v/v) (10 cm³). In the obtained elute the value of pH was adjusted to about 3 using 1M NaOH (GBowniak *et al.*,1996; Lampart-Szczapa *et al.*, 2003). After purification by SPE, recovery of the phenolic acid standards amounted to 96.7% for the vanillin and ferulic acids, 98.2% for the chlorogenic acid and 99.7% for the remaining phenolic acids.

Determination of the phenolic acid composition

Separation and identification of free phenolic acid was carried out using high performance liquid chromatography (HPLC – Waters Milford, MA, USA). For separation, the NovaPak®C_{18} column was used (3.9 x 150 mm; 5 µm). The mixture of methanol [A] and 2.5% water solution of CH_3COOH [B] was used as a mobile phase. Gradient was used during the first 10 minutes when concentration A was linearly increasing from 0 to 10%; during the next 8 minutes it increased up to 20% and during further 22 minutes – up to 70%. The separated compounds were detected at the wave length of 250 and 320 nm (UV-VIS Waters detector). Qualitative identification and quantitative determination of phenolic acids were carried out by comparison of the retention times and peak surfaces of the examined compounds with retention times and peak surfaces of the corresponding standards.

Statistical analysis

Statistical analysis was carried out using the Statistica 7.1 program (StatSoft. Inc., Tulsa, OK, USA). All analyses were repeated at least three times; average results were adopted and standard deviations were calculated. In the analysis, single factor analysis of variance (ANOVA) was applied. When significant differences were noted, the post-hoc analysis was carried out with application of the Tukey's test in order to establish the homogenous groups. To analyse relationship between the studied characteristics, the Pearson linear correlation index was applied.

Results and Discussion

Phenolic compounds play various roles in plants. They are substrates in biosynthesis, protect plants against harmful UV radiation, serve as inhibitors of cellulases produced by pathogens, and they attract pollinating insects (Dixon and Paiva, 1995; Shetty and McCue, 2003). Total content of phenolic compounds is presented in figs.1 and 2. During flower formation, the total content of phenolic compounds in leaves amounted to ca 1400 mg/100 g d.m. for both Kana and Spencer varieties (fig. 1).

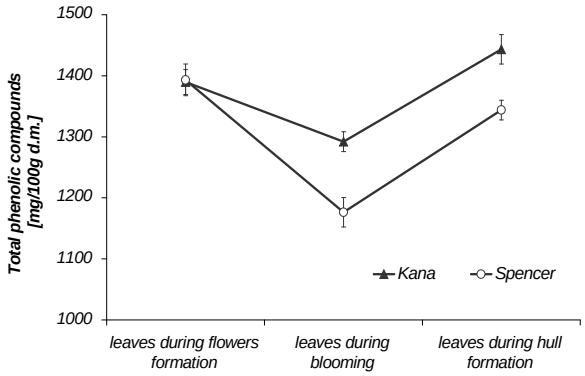

Figure 1: Total phenolic compounds in the rape leaves during successive stages of the plant development

During blooming, total phenols in leaves decreased (by 7% in Kana and by 15% in Spencer), and then it increased by 12% and 14% for Kana and Spencer, respectively (fig. 2). The total of phenolic compounds was higher in the flower primordia and flowers, than in leaves; in Kana, the content exceeded 2095 mg/100 g d.m., and 1808 mg/100 g d.m in Spencer. Significantly lower level was determined in the hull – 1207 mg/100 g d.m. (Kana) and 837 mg/100g d.m. (Spencer). In the rape seeds, the increase of total phenolic compounds was noted (Fig. 2).

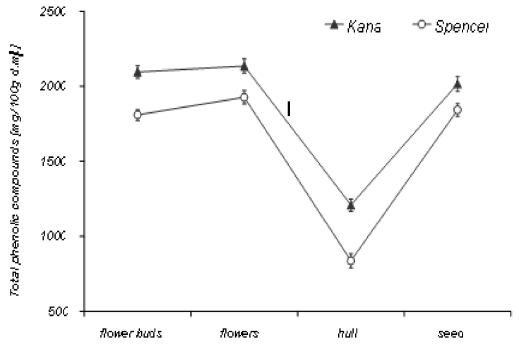

Figure 2: Total phenolic compounds in the rape flower primordia, flowers, hull and seed

Similar relationships were found for the total content of phenolic compounds, (fig. 3, 4). During ripening of the rape plants, content of phenolic acid in leaves during blooming and in hulls decreased.

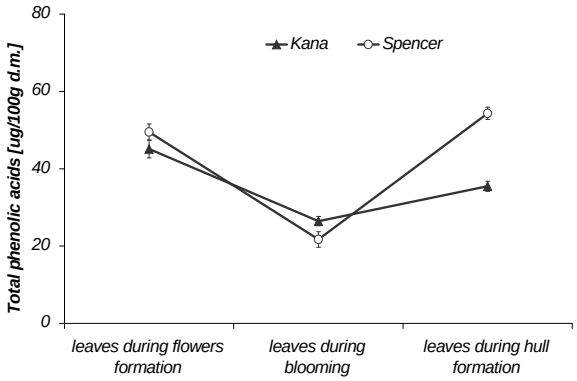

Figure 3**:** Total phenolic acids in the rape leaves in different stages of the plant development

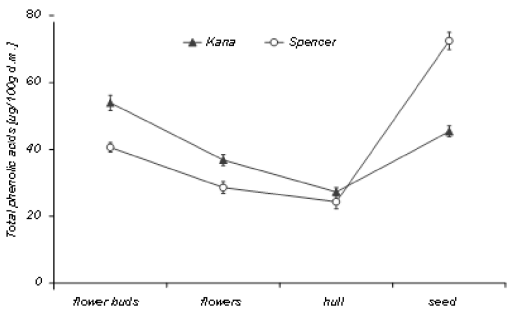

Figure 4: Total phenolic acids in the rape flower primordia, flowers, hull and seed

Decrease of the sinapic acid content during vegetation was noted. During blooming, this decrease in leaves amounted to 54% for Kana and 31% for Spencer. During hull formation, further decrease occurred in leaves by 92% in both varieties. In both cases, the decrease of the sinapic acid content was statistically significant ($p<0.0001$). At the same time, reverse correlation was found for the derivative of the sinapic acid – its content increased during vegetation.In Kana, the content of the sinapic acid derivative in leaves increased over 7 times during hull formation, while in Spencer it increased 14 times (tables 1 and 2). Flower buds had higher content of individual phenolic acids when compared to flowers and hull (table 2). Like in leaves, the sinapic acid and its derivative occurred in greatest quantities, which is characteristic of the rape (Zadernowski and KozBowska, 1983; KozBowska *et al.*, 1990, Lacki and Duvnjak, 1998, Zukalova and Vaak, 1999; Thiyam *et al.*, 2004). From flower primordia, through flowers and hull to seed, a statistically significant ($p<0.0001$) increase of the caffeic acid content was found in both rape varieties. As regards the sinapic acid, its decrease is noted in flowers and then increase during further crop growth. Regarding the derivative of this acid, five fold increase of its content was noted in flowers and decrease in hull down to the quantity found in flower buds. In the seed of both Kana and Spencer, a statistically significant ($p<0.0001$) increase up to the level exceeding 30 mg/100 g d.m. can be noticed (table 2). Phenolic compounds play an important role in the interaction between the plant and the surrounding environment. They can attract insects, have the function of signalling between plants (allelopathy or antibiosis) and between plants and symbiotic bacteria or pathogens (phytopathology), protect plants against the biotic (for example, microbiological or herbivorous vermin) or abiotic (among other things, polluted air, ions of heavy metals, UV-B radiation) stress (Strack, 1997, Bennet and Wallsgrove, 1994, Dixon and Paiva, 1995). Phenylpropanoids (derivatives of the hydroxycinnamon acid) and flavonoids normally accumulate in central vacuoles of the guard cells in the cuticle and subepidermal cells of leaves and

Table 1: Content of phenolic acids in the rape leaves during successive stages of the plant development

Acid	Phenolic acids content [mg/100g d.m.]*					
	Kana			Spencer		
	Leaves during flower formation	*Leaves during blooming*	*Leaves during hull formation*	*Leaves during flower formation*	*Leaves during blooming*	*Leaves during hull formation*
protocatechuic	0.14 ± 0.02^{a}	0.18 ± 0.02^{a}	0.14 ± 0.01^{a}	0.18 ± 0.01^{a}	0.41 ± 0.05^{b}	0.15 ± 0.04^{a}
p-hydroxybenzoic	5.80 ± 0.13^{c}	5.30 ± 0.08^{b}	2.30 ± 0.08^{a}	13.79 ± 0.06^{c}	0.86 ± 0.04^{a}	2.11 ± 0.05^{b}
vanillic	3.52 ± 0.07^{b}	5.72 ± 0.08^{c}	0.80 ± 0.07^{a}	1.30 ± 0.08^{b}	2.07 ± 0.10^{c}	0.57 ± 0.11^{a}
caffeic	0.91 ± 0.05^{b}	0.32 ± 0.05^{a}	1.31 ± 0.03^{c}	1.39 ± 0.08^{b}	0.27 ± 0.03^{a}	1.28 ± 0.11^{b}
p-cumaric	1.13 ± 0.08^{c}	0.61 ± 0.04^{b}	0.45 ± 0.04^{a}	0.18 ± 0.01^{a}	0.70 ± 0.11^{c}	0.39 ± 0.08^{b}
ferulic	3.07 ± 0.11^{c}	0.81 ± 0.04^{a}	1.07 ± 0.05^{b}	3.89 ± 0.09^{c}	1.79 ± 0.05^{b}	0.97 ± 0.07^{a}
sinapic	14.75 ± 0.12^{c}	6.78 ± 0.05^{b}	1.16 ± 0.10^{a}	13.02 ± 0.02^{c}	9.04 ± 0.11^{b}	1.08 ± 0.06^{a}
sinapic acid derivative	4.94 ± 0.08^{a}	12.61 ± 0.05^{b}	36.88 ± 0.07^{c}	3.45 ± 0.07^{a}	6.89 ± 0.08^{b}	47.99 ± 0.20^{c}

* values denoted by different letters differ significantly at the significance level $\alpha = 0.05$

Table 2: Content of phenolic acids in the rape flower primordia, flowers, hull and seed during successive stages of the plant development

Acid	Phenolic acids content [mg/100g d.m.]*							
	Kana				Spencer			
	Flower buds	Flowers	Hull	Seed	Flower buds	Flowers	Hull	Seed
protocatechuic	3.28 ± 0.06^{c}	0.06 ± 0.01^{a}	0.13 ± 0.02^{a}	0.52 ± 0.08^{b}	0.50 ± 0.03^{c}	0.18 ± 0.01^{a}	0.06 ± 0.02^{a}	0.36 ± 0.04^{b}
p-hydroxybenzoic	12.22 ± 0.11^{c}	1.26 ± 0.04^{b}	1.24 ± 0.01^{b}	0.77 ± 0.03^{a}	5.65 ± 0.05^{d}	3.19 ± 0.06^{c}	1.78 ± 0.08^{b}	0.26 ± 0.01^{a}
vanillic	14.27 ± 0.21^{d}	0.36 ± 0.01^{a}	0.82 ± 0.03^{b}	5.81 ± 0.10^{c}	3.50 ± 0.04^{b}	0.30 ± 0.01^{a}	0.20 ± 0.04^{a}	0.45 ± 0.05^{a}
caffeic	0.13 ± 0.01^{a}	0.44 ± 0.04^{b}	1.58 ± 0.08^{c}	1.72 ± 0.08^{c}	0.30 ± 0.03^{a}	0.47 ± 0.03^{a}	1.10 ± 0.03^{b}	5.50 ± 0.12^{c}
p-cumaric	3.28 ± 0.06^{d}	2.93 ± 0.01^{c}	0.75 ± 0.03^{b}	0.09 ± 0.02^{a}	5.17 ± 0.07^{d}	2.91 ± 0.08^{c}	1.45 ± 0.06^{b}	0.30 ± 0.07^{a}
ferulic	4.07 ± 0.07^{d}	3.31 ± 0.06^{c}	1.26 ± 0.06^{b}	0.10 ± 0.04^{a}	11.08 ± 0.08^{c}	1.81 ± 0.06^{b}	1.02 ± 0.06^{a}	0.91 ± 0.05^{a}
sinapic	11.85 ± 0.15^{b}	7.75 ± 0.10^{a}	16.91 ± 0.06^{c}	22.29 ± 0.23^{d}	11.12 ± 0.08^{b}	4.60 ± 0.11^{a}	16.21 ± 0.30^{c}	27.38 ± 0.65^{d}
sinapic acid derivative	4.66 ± 0.10^{a}	20.59 ± 0.18^{b}	4.67 ± 0.13^{a}	31.58 ± 0.12^{c}	3.37 ± 0.11^{a}	15.59 ± 0.27^{b}	3.29 ± 0.06^{a}	38.25 ± 0.36^{c}

* values denoted by different letters differ statistically at the significance level $\alpha = 0.05$

in plant shoot (Moskovitz and Hrazdina, 1981, Parr and Bolwell, 2000). Moreover, some compounds are covalently bound to the plant cell wall while others accumulate on external surfaces of plant parts (Hutzler *et al.*, 1998). In literature there is no data regarding the content of phenolic compounds in green parts of the rape plant. Similar studies were carried out on olive trees in which the phenolic profile was investigated, among other things, in flower buds and leaves (Akillioglu and Tanrisever, 1997). Phenolic profile in leaves differed significantly from that in flower buds. Out of 50 phenolic compounds determined in extracts, 24 were characteristic of flower buds, 30 of leaves, and 5 were common for both (Akillioglu and Tanrisever, 1997). Such differences suggest that in leaves and flower buds these compounds have a clear metabolic function. In leaves, a large number of phenols turned out to be the hydroxycinnamon acid derivatives which are known to be precursors of

the lignin biosynthesis activating or inhibiting the growth of olives. It is highly probable that a phenolic compound in olive leaves directly influences plant growth (Ryan, 2002).

Phenolic acid profile in the seed of the investigated rape varieties is characterised by the greatest content of the sinapic acid and its low-molecular derivative, which was greater in the Spencer variety. These results confirm numerous reports on the content of phenolic acids in the rape seed (KozBowska *et al.*, 1983a, KozBowska *et al.*, 1983b, Zadernowski, 1987, Siger *et al.*, 2004). The research carried out by Rotkiewicz *et al.* (1992) proved that the amount of phenolic acids increased during ripening of the hull reaching the maximum in the harvested seed. This study was carried out for only phenolic compounds and is a part of a larger project regarding the native bioactive components (tocochromanols, beta-carotene, phenolic acids) changes in different parts of plant during its growth. Some results concerning lipophilic compounds were already published (Nogala-KaBucka *et al.*, 2002).

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