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Changes of tocopherol and plastochromanol-8 contents during growth of the spring oilseed rape plant (*Brassica napus* L.)

Zmiany zawartości tokoferoli i plastochromanolu-8 podczas wzrostu rzepaku jarego (*Brassica napus* L.)

Key words: oilseed rape plant, tocopherols, plastochromanol-8, *Brassica napus* L.
Słowa kluczowe: roślina rzepaku, tokoferole, plastochromanol-8, *Brassica napus* L.

Wiele prac badawczych w ostatnim czasie dotyczy biosyntezy chromanoli oraz tokoferylochinonów w komórkach różnych roślin zielonych, gdyż może mieć to zasadnicze znaczenie dla ich występowania w nasionach szczególnie roślin oleistych. Problemem badawczym w niniejszej pracy było określenie zmian w zawartości tokoferoli w wybranych częściach zielonych rośliny i kwiatostanu rzepaku w czasie jego wegetacji oraz w dojrzałych nasionach. Podczas wegetacji roślin rzepaku (odmiana Star) zbierano części roślin (liście, zawiązki kwiatów, kwiaty, łuszczyny) i poddawano liofilizacji. W próbach oznaczano zawartość tokoferoli i plastochromanolu-8 stosując metody chromatograficzne — TLC i HPLC. W przeprowadzonych badaniach stwierdzono występowanie wszystkich czterech homologicznych form tokoferoli i plastochromanolu-8 (PC-8). Najbardziej rozpowszechnionymi okazały się alfa-tokoferol i gamma-tokoferol. W mniejszych ilościach występowały delta- i beta-tokoferol. Stwierdzono również obecność plastochromanolu-8 we wszystkich próbach. Zidentyfikowane w poszczególnych próbach zmienne ilości nagromadzonych tokoferoli i plastochromanolu-8 potwierdzają możliwość zachodzenia biosyntezy tych związków z form niższych przez podstawianie grup metylowych w wolnych pozycjach pierścienia chromanolu.

Recently numerous studies have concerned the biosynthesis of chromanols and tocopherylquinones in cells of various green plants as this can be of crucial importance for their occurrence in seeds, particularly those of oil plants. The present study focused on determination of the changes in tocopherol content in selected green parts and flowers of the oilseed rape plant during growth and in ripe seeds. During growth of the Star variety (spring rapeseed, 00 type) parts of the plant were collected (leaves, buds, flowers, pods) and lyophilized. Then the samples were analysed on the contents of tocopherols and plastochromanol-8 using TLC and HPLC. Occurrence of all four homologous forms of tocopherols as well as plastochromanol-8 (PC-8) was stated. Alfa-tocopherol and gamma-tocopherol were the most abundant compounds observed. Delta- and beta-tocopherol occurred in smaller quantities. The presence of plastochromanol-8 was found in all samples. Varying quantities of tocopherols and plastochromanol-8 identified in individual samples may indicate a possibility of these compounds biosynthesis from lower forms by introducing methyl groups in the free positions of the chromanol ring.

Introduction

Tocopherols are the compounds which naturally occur in green parts of plants and in the seeds. The most important lipid soluble antioxidants in cell membranes are tocopherols. There are four homologous tocopherols: alpha-, beta-, gamma- and delta-. The alpha-tocopherol is known as vitamin E. Tocopherols, first of all alpha-tocopherol, are localized in plastids, especially in chloroplasts of plant cells (Dunphy et al. 1966, Camara et al. 1983, Joyard et al. 1991, Arango and Heise 1998).). Therefore tocopherols have been identified in all photosynthesizing plants with the biggest occurrence in green leaves (Schultz and Soll 1980, Soll et al. 1985).

Potential precursors of biosynthesis of the aromatic ring of tocopherols are shikimic acid and homogentisic acid, while the 20 carbon phytol side-chain is formed after condensation of geranyl-geranyl phyrophosphate (Janiszowska 1986, Janiszowska and Pennock 1976, Furuya et al. 1987). After biosynthesis of the aromatic ring and the side-chain and condensation of both factors and cyclization to the chromanol form the delta-tocopherol is obtained (*tocopherol pathway*). After methylation of the chromanol ring of delta-tocopherol, in green parts of plants, the next tocopherols are formed. Janiszowska and Korczak (1980) obtained several cellular sub-fractions from marigold leaves. They stated that all alpha-tocopherol occurred in plastids (mainly in chloroplasts and chromoplasts). Gamma- and delta-tocopherols occurred in the mitochondrial and microsomal fractions as well as in chloroplasts. Another way of biosynthesis of tocopherols is *tocotrienol pathway.* First of all delta-tocotrienol is generated and afterwards during the following methylation reactions (in which very reactive and high energetic S-adenosine methionine (S-AM) participates as a donor of a methyl group) — other tocotrienols are formed and after that converted to tocopherols (Schultz and Soll 1980, Elmadfa and Bosse 1985, Elmadfa et al. 1989).The last stage of tocopherol biosynthesis is alpha-tocopherol occurring only in the D (+) form. During chemical synthesis only racemic forms of all tocopherols are obtained (Wallat et al. 1986). Till now it has not been univocally proved that biosynthesis can be possible in other organelles of plant cells outside the chloroplasts. However, in many publications authors found factors which had influence on the growth of plants and biosynthesis of tocopherols, for example: light intensity, time of exposure to light and temperature (Furuya et al. 1987, Arango and Heise 1998, Goffman and Mollers 2000).

Because the literature data regarding relation between quality and quantity of biosynthesis of homologous tocopherols in rape plant are incomplete, we focused our studies on investigations of their contents in selected parts during growth of oilseed rape plants, in their flowers and in the seeds.

Materials and Methods

Samples of biological material: leaves, buds, flowers, pods without seeds and seeds of the spring oilseed rape cultivar of *Star* (00 type), were collected during growing period from the experimental fields of Poznan Agricultural University.

First samples were collected from the oilseeds rape plant after 66 days from the sowing. The next collecting took place after 80 days and the following after 95, 110 and 149 days. The last samples were seeds of rape which were technologically ripe.

Standards of alpha-, beta-, gamma- and delta-tocopherol (Merck- Darmstadt, 99.9%) were obtained commercially.

Reagents and organic solvents were of analytical grade (>98%). Peroxide free diethyl ether and silica gel G-60 (Merck-Darmstadt) were used in the investigations.

After collecting fresh green and yellow parts of plants were frozen and immediately lyophylized using a Hetosicc Freeze Dryer apparatus, type Fd 3, Id: 87215. Samples prepared in this way were stored in firmly closed glass flasks, under N_2 without exposure to light. Thin layer of the seeds of rape was slightly dried at 40°C for 1 h, and the seeds were stored in paper bags.

The samples were saponified in order to determine the tocopherol and plastochromanol-8 contents using 60% KOH, and then extracted using peroxide free diethyl ether (Dunphy et al. 1966). After extraction ether was distilled off and in the residue (unsaponifiable matter) tocopherols and plastochromanol-8 were qualitatively and quantitatively determined using TLC (Olejnik et al. 1997, Burgeois 1992) and HPLC methods (Gogolewski et al. 2000).

The TLC separation was made on silica gel G-60 covering glass plates (20 × 20 cm). Solvent system for preparative chromatography was $CHCl_3$. For rechromatography of plastochromanol-8 and alpha-tocopherol the mixture of n-hexane and diethyl ether (95 : 5 v/v) was used as a mobile phase.

Tocopherols were determined by the HPLC method (Gogolewski et al. 2000, Nogala-Kalucka 1999). The HPLC system consisted of the gradient pump Waters Model 600, column, fluorimetric detector and Waters Millenium data acquisition system. Samples dissolved in n-hexane were injected on the LiChrosorb Si 60 column (200 mm, 5 μm Merck), and the mixture of n-hexane and 2-propanol (99.5 : 0.5 v/v) was used as a mobile phase. The flow rate was 1.5 ml/min. The concentrations were calculated from calibration curves made for individual tocopherols.

The results were subjected to statistical analysis. Analyses were carried out in three replicates — each repeated twice. Statistical analysis was conducted using the computer programs Excell and Statgrafic (Manugistic. Inc.).

Results and discussion

Up to now the problem of biosynthesis of tocochromanols and plastochromanol-8 in growing plants has not been fully verified. Many investigations stated that in different parts of a plant, different quantities of these compounds occurred during growth. Some of them may be necessary for growth as they participate in metabolism during ripening of seeds. This refers especially to the oil plants (Schultz and Soll 1980, Janiszowska 1986).

In all parts of the investigated oilseeds rape plant in which tocopherols were determined their four homologues: alpha-, beta-, gamma- and delta-tocopherol and plastochromanol-8 were identified. The results are shown in Table 1 and 2.

The greatest amount of homologous tocopherols was identified in leaves of a plant when the stem was being formed and the height of the plant was ca. 15–20 cm. During blooming of the plant that quantity was lower (about 12.5 mg). During formation of seeds, amount of tocopherols increased to the same level as observed in the first samples collected at the beginning of the stem formation (Table 1). The greatest amount of tocopherols was discovered in buds, a little less – in flowers and the smallest amount- in pods.

Contents of plastochromanol-8 in leaves during the plant growth was much lower than that of tocopherols (it decreased from 9.5 to 1.2 mg). The level of plastochromanol-8 in leaves collected during the time when the stem was being formed was highest (Table 1). Nearly half of this amount was discovered in seeds. Therefore, on the basis of these results one could not univocally state how plastochromanol-8 participated in metabolism and what its biological function in plants was. In many oils e.g., linseed and cornseed (Goffman and Mollers 2000), and also in latex from *Hevea brasiliensis* plastochromanol-8 is present (Whittle et al. 1965).

It is a more effective antioxidant than alpha-tocopherol (Olejnik et al. 1997), but its function is not clear; we only know that plastochromanol-8 can form special complexes with proteins in plant cells. Maybe plastochromanol-8 is used in different reactions but it can decompose under special, inconvenient conditions during the plant growth (e.g. light, temperature, water etc.) (Furuya et al.1987).

From among selected anatomic parts of the rape plant the smallest amount of tocopherols was found in dry pods without seeds (Table 2). Greater quantity was found in flowers and the greatest – in buds (12mg/100 g dm.). Statistical analysis carried out for individual samples proved that standard deviation for tocopherols amounted to 0.22 and for plastochromanol-8 – 0.31 (these results refer to analyses carried out with internal standards).

Table 1

Tocopherol contents in leaves of rape plant during growth period — *Zawartość tokoferoli w liściach w czasie wzrostu rośliny rzepaku*

Sample	Tocopherol								total mg/100 g dm.	PC-8 mg/100 g dm.
	alpha-T		beta-T		gamma-T		delta-T			
	mg/100 g dm.	%	mg/100 g dm.	%	mg/100 g dm.	%	mg/100 g dm.	%		
Leaves during stem formation	16.1[a] ± 0.2 (a)	31.2	1.7[a] ± 0.03 (a)	3.2	28.3[a] ± 0.1 (a)	54.9	5.5[a] ± 0.1 (a)	10.7	51.6[a]	9.5[a] ± 0.3 (a)
Leaves during blooming	16.5 ± 0.1 (b)	42.2	0.3 ± 0.01 (b)	0.7	17.7 ± 0.2 (b)	45.2	4.6 ± 0.1 (b)	11.9	39.1	1.2 ± 0.1 (b)
Leaves during seeds formation	16.9 ± 0.2 (c)	34.4	0.2 ± 0.03 (c)	0.4	19.9 ± 0.8 (c)	40.6	12.1 ± 0.3 (c)	24.6	49.0	1.2 ± 0.1 (c)

[a] Data presents mean values from three replicates and standard deviations at $p \leq 0.05$. Mean values followed by different letter are statistically significant at $p \leq 0.05$

[a] *Dane przedstawiają wartości średnie z trzech powtórzeń oraz odchylenie standardowe $p \leq 0{,}05$. Średnie oznaczone różnymi literami różnią się statystycznie istotnie przy $p \leq 0{,}05$.*

Table 2

Tocopherol contents in selected anatomic parts of rape plant — *Zawartość tokoferoli w wyselekcjonowanych anatomicznych częściach rośliny rzepaku*

Sample	Tocopherol								total mg/100 g dm.	PC-8 mg/100 g dm.
	alpha-T		beta-T		gamma-T		delta-T			
	mg/100 g dm.	%	mg/100 g dm.	%	mg/100 g dm.	%	mg/100 g dm.	%		
Buds	6.0[a] ± 0.2 (a)	50.5	0.2[a] ± 0.02 (a)	1.7	5.6[a] ± 0.1 (a)	46.9	0.1[a] ± 0.2 (a)	0.9	12.0[a]	2.0[a] ± 0.2 (a)
Flowers	8.1 ± 0.1 (b)	75.6	0.6 ± 0.1 (b)	5.5	1.7 ± 0.02 (b)	15.7	0.3 ± 0.1 (b)	3.2	10.7	1.2 ± 0.2 (b)
Pods	1.4 ± 0.1 (c)	38.6	0.03 ± 0.0 (c)	0.8	2.2 ± 0.2 (c)	59.5	0.04 ± 0.2 (a)	1.1	3.7	1.7 ± 0.1 (c)
Seeds	11.1 ± 0.1 (d)	36,5	0.2 ± 0.2 (a)	0,7	18.9 ± 0.2 (d)	62,2	0.2 ± 0.2 (c)	0,6	30.4	4.0 ± 0.1 (d)

[a] Data presents mean values from three replicates and standard deviations at $p \leq 0.05$. Mean values followed by different letters are statistically significant at $p \leq 0.05$

[a] *Dane przedstawiają wartości średnie z trzech powtórzeń oraz odchylenie standardowe $p \leq 0,05$. Średnie oznaczone różnymi literami różnią się statystycznie istotnie przy $p \leq 0,05$.*

Among homologous tocopherols the level of delta-tocopherol in leaves increased during the plant growth, until the time when the seeds where formed, from 10.7 to 24.6%, while its amount in seeds was smallest and it was 0.6%. The quantity of the dimethyltocol derivative (gamma-tocopherol) is greatest in leaves during stem formation; considerable quantities can be seen also in buds and pods. Gamma-tocopherol occurs mainly in seeds (62%), and its antioxidant activity can be observed particularly during their storage. The same tendency was discovered for another derivative of dimethyltocol (beta-tocopherol). Biosynthesis of beta-tocopherol during each stage of evolution of the rape plant was the lowest and its content amounted to only 5% in flowers. It is stated in literature that forms which have free postion 5 — in the chromanol ring more easily react then forms with free postion 7. Only the wheat germs oil is an exception being rich in this tocopherol (Ball 1988, Kamal-Eldin and Appelqvist 1996).

The level of alpha-tocopherol in leaves was varying, and it was observed that it was greatest during blooming (42%); also in flowers the highest level of this homologue was stated. Factors affecting the level of tocopherols are temperature and light considerably stimulating the biosynthesis during growth of the plant (Janiszowska and Jasińska 1982).

The results obtained do not permit univocal conclusions concerning metabolism of tocopherols during growth of the rape plant. It is possible only to note their content in green parts – leaves- in which their high level was discovered. The acquired data can not be discussed in full in relation to other reports due to the lack of literature concerning the quantitative biosynthesis and further metabolism of tocopherols during the plant growth. Majority of studies refer to tocopherol biosynthesis in chloroplasts, and the possibility of biosynthesis occurring in cytoplasm is still under investigation (Janiszowska 1986).

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