Phytochemistry, 1972, Vol. 11, pp. 2581 to 2583. Pergamon Press. Printed in England.

SHORT COMMUNICATION

CAROTENOIDS IN PETALS OF *MEDICAGO FALCATA*

T. IGNASIAK and K. LESINS
Department of Genetics, University of Alberta, Edmonton, Canada

(*Received* 25 *February* 1972)

Key Word Index—*Medicago falcata*; Leguminosae; carotenoids; xanthophyll ester.

Abstract—Petals in three strains of *M. falcata* L. contain lutein 50–52%, lutein-5,6-epoxide 16–18%, chrysanthemaxanthin 9–10%, flavoxanthin 7–8%, and in smaller amounts β-carotene, ζ-carotene, a hydroxy-α-carotene-like pigment, two neoxanthins, auroxanthin and a flavoxanthin-like pigment. Almost 98% of all carotenoids were xanthophylls esterified with fatty acids. Uniformity of major carotenoid content in the three strains is consistent with their all belonging to the same species.

INTRODUCTION

IN THE genus *Medicago*, Simon[1] and Simon and Goodall[2] attempted a chemotaxonomic grouping of species on the basis of the content of phenolics in leaves. Carotenoids in *M. sativa* L., cultivated alfalfa have been investigated by Bickoff *et al.*[3] in dehydrated and fresh vegetative material, while carotenoids in petals of yellow-flowered *M. falcata* have been briefly studied by Cooper and Elliott.[4]

Thus, a detailed analysis of carotenoids in petals of different *Medicago* species might disclose relationships between taxa not readily apparent by other methods. *M. falcata* was chosen since it contains a great variety of different forms often considered as separate species; uniformity or differences in carotenoid content might support or tend to disprove its separation into different taxonomic units.

RESULTS

Pigments were first separated into four fractions on an alumina column. These fractions were subjected to further purification and identification in different TLC systems, to spectrophotometric analysis, and to some additional colour tests.

Fraction I was eluted from an alumina column with ether. It was entirely epiphasic on partition test with 95% methanol–petroleum. By TLC on MgO-plates with 50% benzene in petroleum two pigments were isolated. On comparing their spectra and chromatographic properties with data from the literature[5,6] and co-chromatographing with authentic β-carotene they were identified as β-carotene and ζ-carotene.

Fraction II was eluted with 0·5% ethanol in ether. It showed partly hypophasic properties on shaking with 95% methanol–petroleum. TLC on MgO-plates with 30% acetone in

[1] J. P. SIMON, *Austral. J. Bot.* **15,** 83 (1967).
[2] J. P. SIMON and D. W. GOODALL, *Austral. J. Bot.* **16,** 89 (1968).
[3] E. M. BICKOFF, A. L. LIVINGSTON, G. F. BAILEY and C. R. THOMPSON, *J. Agric. Food Chem.* **2,** 563 (1954).
[4] R. L. COOPER and F. C. ELLIOTT, *Crop Sci.* **4,** 367 (1964).
[5] T. W. GOODWIN, *Biochem. J.* **50,** 550 (1952).
[6] A. HAGER and T. MEYER-BERTENRATH, *Planta Berl.* **76,** 149 (1967).

petroleum or 2% methanol in benzene gave only one band. The visible spectrum resembled that of α-carotene and since its absorption affinity on aluminum and magnesium oxides was different from that of α-carotene and lutein, it evidently is a monohydroxy-α-carotene.

Fraction III was eluted with 5% ethanol in ether. It was entirely hypophasic. On separation on $CaCO_3$-layer with 30% acetone in petroleum it gave two broad zones. The zone of R_f 0·43–0·66 was rechromatographed on MgO-plate with 30% acetone in petroleum. The three fine bands obtained were identified as lutein, lutein-5,6-epoxide and violaxanthin on the basis of their absorption maxima[6,7] and their R_fs on reversed phase partition TLC.[7,8] In addition, lutein-5,6-epoxide and violaxanthin in ethanol with a few drops of 0·1 N HCl changed their spectra to those of flavoxanthin and auroxanthin; isomerization to furanoid derivatives in the presence of a trace of HCl is characteristic for 5,6-epoxides.[6,9] The second zone of R_f 0–0·20 was treated on $CaCO_3$-layer with a mixture of petroleum, acetone and chloroform. It was separated into two pigments, identified as chrysanthemaxanthin and flavoxanthin. Spectra of chrysanthemaxanthin and flavoxanthin are almost identical;[10] the main criterion used to distinguish them is a colour reaction with 20% HCl. An ether solution of flavoxanthin gives a blue colour with 20% HCl, whereas that of chrysanthemaxanthin is unaffected.[10]

TABLE 1. QUANTITIES OF CAROTENOIDS IN PETALS OF DIFFERENT *M. falcata* STRAINS

Carotenoid	Acc. No. 127 Dry matter (μ/g)	%	Acc. No. 137 Dry matter (μ/g)	%	Acc. No. 497 Dry matter (μ/g)	%
β-Carotene	14·2	1·4	11·1	1·2	5·1	0·9
ζ-Carotene	2·0	0·2	1·6	0·2	0·6	0·1
Hydroxy-α-carotene-like	7·0	0·8	8·3	0·9	7·4	1·3
Lutein*	546·0	52·0	459·0	50·0	290·0	50·0
Lutein-5,6-epoxide*	162·5	15·5	134·6	14·7	104·0	18·2
Violaxanthin*	112·0	10·5	90·0	9·9	43·6	7·6
Neoxanthin, all-*trans*	27·6	2·7	32·8	3·6	13·2	2·3
Neoxanthin, 9-*cis*	4·9	0·5	4·6	0·5	1·9	0·3
Chrysanthemaxanthin	90·5	8·5	93·5	10·2	49·5	8·6
Flavoxanthin*	78·0	7·4	72·0	7·9	37·8	6·6
Flavoxanthin-like	—	—	—	—	3·4	0·5
Auroxanthin	4·7	0·5	7·9	0·9	21·2	3·7
Total	1049·9	100·0	915·4	100·0	577·7	100·0

* In different isomeric forms.

Fraction IV was eluted with 50% ethanol in ether. On $CaCO_3$-layer with 30% acetone in petroleum, three clear bands of R_f 0·30, 0·34, 0·58 and one diffuse zone between 0 and 0·20 were obtained. The pigment of the highest R_f showed all properties of violaxanthin. The two others were identified as neoxanthin all-*trans* and one of its isomers, likely the 9-*cis*-;[11] on plates of impregnated kieselguhr both pigments had the same R_f, the spots being located above that of violaxanthin which is characteristic for trihydroxymonoepoxides.[7,8] On isomerization with iodine in hexane each pigment gave characteristic chromatographic

[7] K. EGGER, *Planta Berl.* **80**, 65 (1968).
[8] K. EGGER, *Planta* **58**, 664 (1962).
[9] N. I. KRINSKY and T. H. GOLDSMITH, *Arch. Biochem. Biophys.* **91**, 271 (1960).
[10] P. KARRER and E. JUCKER, *Carotenoids*, p. 212 (1950).
[11] L. CHOLNOKY, K. GYÖRGYFY, A. RONAI, J. SZABOLCS, G. GALASKO, A. K. MALLAMS, E. S. WAIGHT and B. C. L. WEEDON, *J. Chem. Soc.* **C**, 1256 (1969).

spots ($CaCO_3$-layer/30% acetone in petroleum).[6] Iodine-catalyzed equilibrium mixture of each pigment had the same visible maxima (466, 436, 413 nm); in the presence of dil. HCl, both gave the same hypsochromic shift. The fourth diffuse zone was rechromatographed on $CaCO_3$-plate with a mixture of petroleum, acetone and chloroform. It gave four bands, three of which could be identified by their spectra and colour reactions with conc. HCl as chrysanthemaxanthin, flavoxanthin and auroxanthin. The spectrum of the fourth pigment showed the shape of flavoxanthin but maxima were slightly shifted to the shorter wavelength hence it was denoted as a flavoxanthin-like pigment.

Quantities of carotenoids are given in Table 1. GLC analysis of methyl esters obtained after saponification of fatty acids showed that the palmitic and linolenic acids were major components, accompanied by some myristic and linolic acids.

DISCUSSION

In all three strains of *M. falcata*, there were some common features. Close to 98% of the total carotenoids was in form of xanthophylls esterified with fatty acids. Half of the carotenoids consisted of lutein; its 5,6-epoxide was the next in amount. Violaxanthin, chrysanthemaxanthin and flavoxanthin were present to the extent of 7–10%. This general uniformity in major carotenoids is to be expected in different strains belonging to the same species. In No. 497 a flavoxanthin-like carotenoid not present in the others was found, and the percentage of auroxanthin in No. 497 was distinctly higher; furthermore the total amount of carotenoids was distinctly lower. It is interesting that this strain is morphologically different. Reported as strain No. 14, syn. *M. erecta* by Lesins and Lesins,[12] it has upright growth habit in contrast to prostrate or semiprostrate in other strains, has a taproot instead of branched roots, and has a dense vegetative pubescence different from other strains.

EXPERIMENTAL

Materials. Three diploid ($2n = 16$) *M. falcata* strains often referred to as separate species were investigated: Acc. No. 127 (*M. falcata*, cultivar Kubanskaya), Acc. No. 137 (Syn. *M. quasifalcata* Sinsk.), and Acc. No. 497 (Syn. *M. erecta* Kotov). Petals were picked from freshly gathered flowers, weighed, and preserved in vials under petroleum at −25° until used.

Extraction of pigments. 5–7 g of petals were macerated and extracted separately with 3 × 25 ml of acetone, Et_2O and petroleum. The extracts were combined, reduced to *ca.* 75 ml and transferred into Et_2O which was washed 3 × ice-cold H_2O.[13] Et_2O was evaporated and the residue saponified. Evaporations were under reduced pressure below 30° in subdued light.

Saponification. The residue in 40 ml EtOH plus 10 ml Et_2O was saponified with 10 ml 60% KOH and worked up by standard procedures.[13]

Column chromatography and TLC. These were carried out using standard procedures.[5,8,14]

Quantitative estimation. 1 g of fr. petals was dried to constant wt at 105°. Absorption was measured for each of the purified pigments. The $E^{1\%}_{1cm}$ values for calculation of quantities were taken from the literature.[13,15]

Analysis of fatty acids. The dry residue of the mixture of fatty acids was esterified in 3 ml Et_2O + 5 ml MeOH, with 0·05 ml of conc. H_2SO_4. The methyl esters in CH_2Cl_2 were analysed by GLC on Hewlett–Packard Chromatograph Model 5750 (with flame ionization detector), equipped with 1·8 m × 2 mm glass column (10% DEGS on 80/100 Chromosorb), operated at 15°/min from 75 to 160° with He (40 ml/min) as carrier gas. Peaks were identified by comparison with authentic samples.

Acknowledgement—These studies were supported by the National Research Council of Canada (Grant No. A-1425).

[12] K. Lesins and I. Lesins, *Can. J. Genet. Cytol.* **6**, 152 (1964).
[13] T. W. Goodwin, in *Methoden der Pflansenanalyse*, Vol. III, p. 272 (1955).
[14] A. Hager, and T. Meyer-Bertenrath, *Planta Berl.* **69**, 198 (1966).
[15] B. H. Davies, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. Goodwin), p. 529 (1965).