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Flavonoids analysis of *Vicia* species of Narbonensis complex: *V. kalakhensis* Khatt., Maxt. & Bisby and *V. eristalioides* Maxt.

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Abstract — A qualitative and quantitative analysis of flavonoids has been carried out for first time in *Vicia eristalioides* Maxt. and in *Vicia kalakhensis* Khatt., Maxt. & Bisby. Free aglycones were consistently absent from both species while kaempferol derivatives were pre dominant in *V. kalakhensis;* a more complex mixture of flavonoid glycosides,(kaempferol and quercetin glycosides) was present in *V. eristalioides.* Therer was no evidence of flavones glycosides.The flavonoid patterns of *V. kalakhensis* and *V. eristalioides* were compared with that of *V. narbonensis* which is considered to be the ancestor of the Narbonensis complex. The results indicate that qualitative and quantitative flavonoid data may be used in the study of the organization and evolution of the Narbonensis complex.

Key words: aglycones, chemosystematics, evolution, flavonoids, glycosides, Narbonensis complex, *Vicia eristalioides, Vicia kalakhensis, Vicia narbonensis*

INTRODUCTION

Many attempts have been made to divide the genus *Vicia* into subgenera and sections. Recently MAXTED *et al.* (1991) proposed a new classification based on that by KUPICA (1976) in which the section Faba was split into three taxa, two of which are monospecific (Taxon A: *V. bithynicca* L. and Taxon C: *V. faba* L.), whereas the third, Taxon B, includes seven species *(V. narbonensis* L., *V. galilaea* Plim et Zoth, *V. hyaeniscyamus* Mouter, *V. kalakhensis* Khatt., Maxt. & Bisby, *V. ertstaliotdes Maxt., V. serratifolia* Jacq. and *V.johannis* Tamamsch). In 1993 the same author (MAXTED 1993) divided Taxon B into two subsections: Rombocarpae and Narbonensis, the former monospecific *(V. eristalioides* Maxt.) and the latter comprising the re maining six species.

Because of their widespread occurrence and chemical stability, flavonoids are well accepted as chemical markers in plant taxonomy as a useful tool for the characterization and classification of higher plants (ASEN 1984; HARBORNE and TURNER 1984; VAN SUMERE *et al.* 1985). In a previous chemosystematic report WEBB and HARBORNE (1991) studied flavonoid aglycones from acid hydrolyzed leave extracts indicating that flavonoid data were meaningful at sectional level and suggested that variations in glycosidic type present in *Vicia* flavonoids could be inter esting from a chemotaxonomic point of view.

In order to study new characters of taxonomic value, the aim of the present work was to identify the flavonoid compounds in two species not previously investigated for glycosides, *V. eristalioides* and *V. kalakhensis,* and in *V. narbonensis* (considered the ancestor of Narbonensis complex) to determine whether flavonoid patterns were meaningful to assist the lat est classification by MAXTED (1993) and could provide a better understanding of the relationship inside Taxon B.

Qualitative and quantitative analyses of flavonoids may be considered as an useful complement to the reports of CREMONINI *et al.* (1989a, b, 1993), FREDIANI *et al.* (1987, 1992), DE PACE *et al* (1991), MAGGINI *et al.* (1991, 1995) based

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on cytological, karyological and biochemical characters in the *Vicia* species.

MATERIAL AND METHODS

Plant material

Seeds of *Vicia kalakhensis* Khatt., Maxt. & Bisby (accession number 877321) and of *Vicia eristalioides* Maxt. (accession number 867095) were kindly provided by the genebank of the Department of Botany of the University of Southampton (U.K.); seeds of *Vicia narbonensis* (accession number 125786) were provided from the Istituto per il Germoplasma C.N.R., Bari.

V. narbonensis (2n=10 and nuclear DNA content: 29.10 pg, CREMONINI *et al.* 1998a) shows a wide distribution in the Mediterranean area, but *V. kalakhensis* (2n=10 and nuclear DNA content: 42.22pg, CREMONINI *et al.* 1998a) and *V. eristalioides* (2n=10 and nuclear DNA content: 38.58 pg, CREMONINI *et al.* 1998a) are only present in restricted areales in Syria and Turkey respectively.

Plantlets were grown up to the sixth whorl of leaves in a grown chamber at 20°C with a 12h light-dark photoperiod without UV light. Fresh plants, without roots, were weighed (69.9g and 78.55 g and 68.0g for *V. kalakhensis V. eristalioides* and *V. narbonensis,* respectively) and immediately freeze-dried.

Extraction of plant material

The lyophilised plant material (6.9g and 7.4g and 6.5g for *V. kalakhensis, V. eristalioides* and *V. narbonensis,* respectively) was refluxed in asoxhlet apparatus with n-exane for 48 h and then extracted in methanol at room temperature four times, each for 72h.

Qualitative analysis

The combined methanolic extracts (2.38g, 2.70g and 2.40g, respectively) were concentrated under vacuum and fractionated by gel filtration on Sephadex LH20 column chromatography (Pharmacia, Uppsala, Germany) using methanol-water (v:v, 1:1) as eluent. The fractions were analysed for flavonoid compounds by HPLC and TLC (SiO_2 Merck, Darmstad, Germany; ethyl-acetate, formic acid, acetic acid, water, v:v:v:v, 10: 1.1: 1.1: 2.7, or Toluene-ethyl-acetate-formic acid, v:v:v, 5:4:1). TLC plates were sprayed with Naturstoffreagenz A (Roth, Karlsruhe, Germany) and polyethyleneglycol 4000 (Merck) for detection of flavonoid compounds.

HPLC analysis was performed with a Waters 600E chromatograph, equipped with a Waters 990 Photodiode Array Detector (Waters, Milford, MA, USA) and a DIOL (5µm) LiChroCART (250x4 mm, Merck) column. The solvent systems comprised of chloroform-methanol-acetonitrile (75:21:4) for glycosides and 100% chloroform for aglycones.

The purified flavonoid fractions were hydrolysed by heating for 1 hr at 100°C in 2N HCl-ethanol (1:1) in stoppered tubes. Aglycones were identified on silica gel TLC (toluene-ethylacetate-formic acid 5:4:1; NTS/PEG) and by HPLC, while sugars on silica gel TLC (chloroform-methanol-water 6:4:1; carbazole-sulphuric acid).

The Rf on silica gel, retention times (min), the U.V. data and hydrolysis products are reported in Table 1.

Quantitative analysis

The method for flavonoid quantitation by the F.U.I., IX ed. (1991), in the monography of *Crataegus monogyna* Jacq. and *Crategus laevigata* (Poiret) DC., synonymous of *Crategus oxycantha* (L.) Jacq., was applied to 33.9mg, 32.6mg and 35.4mg of the methanolic extracts of *V. kalakhensis, V. eristalioides* and *V. narbonensis,* respectively. The absorbance of the samples at 425nm was evaluated. The flavonoids content (expressed as mg quercetin/mg lyophilized plant material) was calculated using a calibration curve obtained with pure quercetin; all the measurements were carried out in triplicate.

RESULTS AND DISCUSSION

Since one of the most frequent doubts concerning the employment of flavonoids as chemical markers is the effect of the environment on their biosynthesis, we selected plants cultivated in controlled conditions and at the same developmental stage and we used the same analysis conditions to point out qualitative and quantitative variability due to genetic variation and we have used light without UV since UV light may influence flavonoids patterns (MARKHAM *et al.* 1998).

Table 2 shows the flavonoid patterns in methanolic extracts of *Vicia kalakhensis, V. eristalioides* and *V. narbonensis.* In *V. kalakhensis,* mainly kaempferol glycosides were present, while quercetin glycosides occured only in trace

TABLE 1 — Rf, Retenction time, UV data and hydrolysis products of flavonoids in *V. kalakhensis* (V.k.), *V. eristalioides* (V.e.) and *V. narbonensis* (V.n.) also after Sephadex separation.

	Rf [SiO2, ethylacetate-formic acid-acetic acid- water 10: 1.1: 1.1: 2.7]	Tr HPLC (min) [DIOL,chloroform-methanol-acetoni trile 75:21:4]	UV (λ max, nm)	Hydrolysis products:		Sephadex LH20 hydrolysed fractions		
				Aglyconess	Sugar	V.k.	V.e.	V.n.
Kaempferol-3-O-rhamnoside	0.80	3.10	266.5, 353.5	Kaempferol (Tr=4.9)	rhamnose (Rf=0.43)	+ (XIX-XX)	+ (XXI-XXII)	—
Kaempferol-3-O-glucoside	0.78	3.89	267.0, 353.0	Kaempferol	glucose (Rf=0.35)	+ (XV)	+ (XI-XIV)	—
Kaempferol-3-O-galactoside	0.64	4.24	257.0, 359.7	Kaempferol	galactose (Rf=0.22)	+ (XVII)	+ (XI-XIV)	—
Kaempferol-3-O-rutinoside	0.49	7.24	259.4, 362.7	Kaempferol	glucose, rhamnose	+ (XXVI)	+ (XVIII-XIX)	+ (XIX)
Quercetin-3-O-rhamnoside	0.76	6.68	257.0, 351.8	Quercetin (Tr=8.65)	rhamnose		+ (XV-XVI)	+ (XX)
Quercetin-3-O-galactoside	0.59	7.3	259.4, 362.7	Quercetin	galactose	+ (XXVI)	—	+ (XIV)
Quercetin-3-O-rutinoside	0.47	11.86	259.4, 360.7	Quercetin	glucose, rhamnose	+ (XXVI)	+ (XVIII)	+ (XX)
Kaempferol-glycoside	0.72	4.65	268.0, 354.	Kaempferol	unknown	+ (XXVI)	+ (XXIV)	—
Quercetin-glycoside	0.65	6.90	267.0, 364.0	Quercetin	unknown	—	+ (XXIV)	—
Quercetin-glycoside	0.54	7.25	267.0, 365.0	Quercetin	unknown	—	+ (XXVI)	—
Quercetin-glycoside	0.21	8.90	267.0, 362.0	Quercetin	unknown	—	+ (IX-X)	—
Quercetin-glycoside	0.40	10.40	273.0, 358.0	Quercetin	unknown	—	+ (X)	+ (XIV)
Quercetin-glycoside	0.12	22.00	262.0, 352.0	Quercetin	unknown	—	—	+ (XVI)

TABLE 2 — HPLC flavonoidic pattern in methanolic extracts of *V. kalakhensis, V. eistalioides* and *V. narbonensis* (Tr= retention time (min), (+) = traces) and flavonoid percentage.

	V. kalakhensis	*V. eristalioides*	*V. narbonensis*
Kaempferol-3-O-rhamnoside	+	+	
Kaempferol-3-O-glucoside	+	+	
Kaempferol-3-O-galactoside	+	+	
Kaempferol-glycoside (Tr=4.65)	+	+	
Kaempferol-3-O-rutinoside	+	+	
Quercetin-3-O-rhamnoside		+	+
Quercetin-3-O-galactoside	(+)		+
Quercetin-glycoside (Tr=6.90)		+	
Quercetin-glycoside (Tr=8.90)		+	
Quercetin-glycoside (Tr=10.4)		+	+
Quercetin-3-O-rutinoside	(+)	+	+
Quercetin-glycoside			+
Flavonoid percentage (mg Qc/mg lyophilized)	0.32	0.31	0.74

amounts; while in *V. eristalioides* a more complex mixture of kaempferol and quercetin derivatives was found and in *V. narbonensis* almost exclusively quercitin glycosides were identified. Furthermore there was no evidence of flavone glycosides (apigenin and luteolin derivatives) in all the methanolic extracts.

HPLC analysis, performed in typical conditions for flavonoid aglycones, showed that free kaempferol, quercitin, myricetin, apigenin, luteolin and chrysoeriol were never present. The results of the quantitative analysis (Table 2) show that the flavonoid percentage (mg Qc/mg lyophilized plant material) in *V. narbonensis* is twice that in *V. eristalioides* and *V. kalakhensis.*

A recent study of flavonoid aglycones present in acid hydrolyzed extracts of leaves of *Vicia* species (WEBB and HARBORNE 1991) showed that six flavonoid aglycones (apigenin, myricetin, kaempferol, luteolin, quercetin and diosmetin) were present. The flavones (luteolin, apigenin and diosmetin), characteristic of subgenus Vicilla and of sections Hypechusa and Peregrinaee (subgenus Vicia) were always ab sent from the Narbonensis complex. WEBB and HARBORNE (1991) identified kaempferol and kaempferol and quercetin from hydrolyzed leaf extracts of *V. kalakhensis* and *V. eristalioides* respectively and no information on glycosides was shown.

The presence of flavonols and the absence of flavones pointed out by WEBB and HARBONE (1991) in the species of the Narbonensis complex is in good agreement with the results reported in Table 2. Moreover our results underline a significant difference between the three species which was not revealed by the previous aglycone analyses conducted only on leaves by WEBB and HARBORNE (1991). Thus the three species are easly recognizable by their glycosidic composition and the qualitative flavonoid analysis could support the splitting of the Taxon B as proposed by MAXTED (1997). The insertion of *V. eristalioides* in the monospecific group of Rhombocarpae is justified by its more complex flavonoid mixture and the absence of quercetin-3-O-galactoside which is, on the con trary, always present together with quercetin-3 - O-rutinoside, in species included in Taxon B (PERRINO *ET AL.* 1989).

Cytophotometric analyses of nuclear DNA of the three species (CREMONINI *et al.* 1998a, b) showed that *V. kalakhensis* was the species with the greatest DNA nuclear content, followed by *V. eristalioides* and *V. narbonensis.* It is well accepted that plant evolution may be represented by different values of nuclear DNA content that involve loss or acquisition of DNA sequences. Redundancy modulation of repeated DNA sequences has been shown to occur within several plant species; intra or interspecific changes in genome size may play a role in environmental adaptation and speciation (FREDIANI *et al.* 1999 and references therein). In agreement with BENNETZEN'S and KELLOG'S hypothesis (1997), (in an analysis of genome size variation in relation to phylogeny in the Poaceae the evolution is accompanied by an increase in genome size) the degree of evolution is greater for *V. kalakhensis,*

followed by *V. eristalioides* and then by *V. narbonensis.*

Studies on *Vicia faba* L., the most evolved species in the section Faba from a cytological point of view, pointed out the presence in this plant of kaempferol glycosides and only traces of quercetin glycosides (VIESTRA *et al.* 1982; WEISSENBOCK *et al.* 1984; TOMAS-LAURENT *et al.* 1989). Moreover, during the emersion of plant from aquatic to terrestrial habitats, the biosynthesis of flavonoid compounds, which protect the plants against UV irradiation, was of fundamental importance from the very beginning (MARKHAM 1988; LES and SHERIDAN 1990). Consequently, from our qualitative and quantitative results on flavonoids (Table 2) we can suppose that evolution, inside Narbonensis complex, preceded from species with larger amount of flavonoids and with more complex structure (3'4' — hydroxy — derivatives) to species with flavonoids having simpler structure (4'-hydroxy-derivatives) and a lower flavonoid content due to a reduced protection necessity against U.V. irradiation. Qualitative and quantitative flavonoid analyses confirm that *V. narbonensis* may be considered the ancestor of the Narbonensis complex and *V. kalakhensis* and *V. eristalioides* may be considered the evolved species even if with two different solutions since *V. eristalioides* shows Qc-glycosides and kae-glycosides and *V. kalakhensis* shows only kae-glycosides. In this connection it may be worth nothing that our conclusion is also supported by the geographical distribution and by morphological, anatomical and molecular description of the analysed species (MAXTED 1988, 1993; KHATTAB *et al.* 1988; MAXTED *et al.* 1993; BENNET and MAXTED 1997; JAASKA 1997; CREMONINI *et al.* 1998a, b; POTOKINA *et al.* 1999; NOUZOVA *et al.* 1999; VENORA *et al.* 2000). Indeed, the present research may be a further step in the study of the relationships between *Vicia* species; work is in progress on the other species of the Narbonensis complex and it may facilitate greater understanding of the evolution inside this complex.

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