6

Flavonoid HPLC fingerprints of wild *Vigna* species

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Abstract

Thirty-two wild species and varieties of *Vigna* of different origin were screened for their flavonoid content. The compounds detected were utilized to assess both inter- and intraspecific relationships. Flavonoid HPLC fingerprints support evidence for the existence of different flavonoid chemotypes, which may reflect differences in geographic origin. As far as interspecific relationships are concerned, species belonging to sections *Vigna, Plectotropis,* and *Ceratotropis* do not show flavonoid glycosides in common with cultivated lines of *Vigna*. By contrast, some relationships have been found between cultivated lines and wild species of section *Catiang*. A greater variability in flavonoid aglycone class and glycosylation pattern has been observed in cultivars of *V. unguiculata* (L.) Walp., compared to the wild species. The taxonomic and ecological significance of these findings is discussed. Finally, the existence of a positive relationship between resistance/susceptibility characteristics against aphids and qualitative and/or quantitative flavonoid content is also discussed.

Introduction

The genus *Vigna* (Leguminosae) contains ~160 species, of which several are economically important crops in the agricultural ecosystem of tropical regions. Although frequently revised by taxonomists, the genus has been divided into 7 subgenera (Maréchal et al. 1978). Two subgenera (*Sigmoidotropis* and *Lasiospron*) are endemic to America, and five subgenera (*Vigna, Haydonia, Plectotropis, Macrorhyncha,* and *Ceratotropis*) are distributed in Africa and Asia (Ng and Maréchal 1985). Due to the presence of several centers of origin and the large morphological diversity, it is difficult to draw intrageneric relationships within *Vigna;* consequently, chemical markers have been used to help in establishing generic relationships (Birch et al. 1986; Rao et al. 1992; Panella et al. 1993; Vaillancourt and Weeden 1993; Zallocchi and Pomilio 1994). As a useful tool for the characterization and classification of higher plants, the importance of flavonoids as chemical markers in plant taxonomy is well documented (Bate-Smith 1966; Harborne 1971; Harborne and Turner 1984; Van Sumere et al. 1985; Bohm 1987; Perrino et al. 1989; Hegnauer and Grayer-Barkmeijer 1993).

In order to evaluate the taxonomic significance of flavonoid occurrence, it is essential to identify, at least partly, the various compounds present. In some cases, it may be sufficient to establish which classes of flavonoids are represented (i.e., the presence or

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absence of flavonols, flavones, or C-glycosylflavones) and differences in hydroxylation, O-methylation, and glycosylation (Bohm 1987). Variations in taxonomic characters may be induced or influenced by environmental factors. However, when changes do occur they can involve level and position of glycosylation, with no alteration of flavonoid aglycone (Bohm 1987); qualitative variability will probably be due to genetic variation, while the quantitative variability will reflect environmental factors.

Flavonoid HPLC fingerprints of *Vigna* leaves have shown considerable promise: there are qualitative and quantitative differences in flavonoid patterns between species and/or accessions (Lattanzio et al. 1990; Lattanzio et al. 1992). In addition, chemical characterization of *Vigna* species is of particular relevance because wild species represent a reservoir of useful genes that could be used in cowpea improvement. Investigation on the levels of resistance of wild species of *Vigna* to pests and diseases showed good levels of resistance, offering promise for their potential use in cowpea breeding (Padulosi and Ng 1990).

In the present study, 32 *Vigna* species and/or accessions were analyzed by HPLC for their leaf flavonoid contents. The flavonoid glycosidic patterns were used to draw intra- and interpecific relationships. The ecological significance of these findings is discussed.

Materials and methods

Most *Vigna* species used in this study (Table 1) were collected in Africa. All seed samples were supplied by the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria.

All species were grown from seed in a growth chamber at 25 °C (70% RH), with a 12h:12h light-dark photoperiod up to emission of the sixth leaf. Second and third leaves from the tip were collected from each plant (5–10 g) and analyzed for phenolic compounds.

For qualitative determination of the flavonoids, the plant material was refluxed with hot methanol-ethanol-water (MeOH-EtOH-H_2O) (4:4:2) for 1 h. After centrifugation, the solution was concentrated under vacuum and partitioned with petroleum ether (bp 40–70 °C). The aqueous fraction was analyzed for flavonoid HPLC fingerprint. For flavonoid aglycone analysis, the same fractions were hydrolyzed under nitrogen with 0.3 M HCl and then extracted with diethyl ether (Et_2O). Finally, the Et_2O extracts were concentrated under vacuum and redissolved in MeOH. The latter were analyzed by HPLC, using a Perkin Elmer Series 4 liquid chromatograph, equipped with a computer-aided spectrophotometric photodiode array detector 1040 Hewlett Packard, following the method of Lattanzio and Van Sumere (1987). In all cases, flavonoids were subjected to UV spectroscopy and chromatographic comparison against authentic samples, by means of a computer program.

From chromatograms and using an HP-K3 software postrun analysis coupled with PE-Chromatographics 2 software, a flavonoid fingerprint of the different *Vigna* species was obtained, based on the retention times of the flavonoid glycosides, knowledge of aglycone, and spectral data.

Results and discussion

Cultivated lines of V. unguiculata *(L.) Walp.*

Flavonoid HPLC analyses clearly showed that cultivated lines of *Vigna unguiculata* (L.) Walp. always contained three flavonoid aglycones: quercetin (the most abundant), kaempferol, and isorhamnetin (Table 1). The flavonoid glycoside patterns of the different

Table 1. Distribution of flavonoid aglycones in *Vigna* species and/or accessions of different origin.

Subgenera/sections/taxa†	Origin	K§	Q	I	A
Vigna					
Catiang					
V. unguiculata (L.) Walp.					
ssp. *unguiculata* cg. *unguiculata* Westphal (c)	Nigeria	+	+++	+	–
ssp. *dekindtiana* var. *dekindtiana* MG 112997 (w)	Nigeria	+	+++	+	–
ssp. *dekindtiana* var. *dekindtiana* TVNu 413 (w)	Nigeria	(t)	+++	–	–
ssp. *dekindtiana* var. *mensensis* (Schweinf.) M.,M.&S.(1) TVNu 862 (w)	Zimbabwe	+++	(t)	–	(t)
ssp. *dekindtiana* var. *protracta* (Wilczek) M.,M.&S. TVNu 965 (w)	Swaziland	+++	+	+	–
ssp. *dekindtiana* var. *pubescens* (Wilczek) M.,M.&S TVNu 110 (w)	Tanzania	+++	(t)	–	–
ssp. *stenophylla* (Harv.) M.,M. and S. TVNu 714 (w)	South Africa	+++	+	–	–
ssp. *tenuis* TVNu 661 (E. Mey.) M.,M.&S. var. *tenuis* TVNu 661 (w)	Congo	+++	(t)	–	–
Vigna					
V. ambacensis Baker var. *ambacensis* TVNu 755 (w)	Central Africa Rep.	+++	+	–	–
V. gracilis Hooker fil var. *gracilis* TVNu 18 (w)	Ivory Coast	+++	(t)	–	–
V. heterophylla A. Richard TVNu 19 (w)	Kenya	+	(t)	+++	–
V. luteola (Jacq.) Bentham TVNu 475 (w)	Kenya	–	+++	–	–
V. luteola (Jacq.) Bentham TVNu 172 (w)	Brazil	+++	(t)	–	–
V. luteola (Jacq.) Bentham TVNu 905 (w)	Botswana	+++	–	–	–
V. marina (Burm.) Merrill var. *oblonga* TVNu 1174 (w)	Gabon	(t)	–	+++	–
V. marina (Burm.) Merrill var. *marina* TVNu 717 (w)	Mozambique	+++	–	–	–
V. oblongifolia A. Richard var. *oblongifolia* TVNu 88 (w)	Nigeria	–	+++	–	–
V. oblongifolia A. Richard var. *oblongifolia* TVNu 40 (w)	Rwanda	–	+++	–	–
V. oblongifolia A. Richard var. *oblongifolia* TVNu 37 (w)	Costa Rica	–	+++	–	–
V. oblongifolia A. Richard var. *oblongifolia* TVNu 135 (w)	Nigeria	–	+++	–	–
V. racemosa Hutch & Dalziel TVNu 181 (w)	Nigeria	+	+++	(t)	–
V. racemosa Hutch & Dalziel TVNu 260 (w)	Central Africa Rep.	+	+++	–	–
V. racemosa Hutch & Dalziel TVNu 96 (w)	Nigeria	+++	+	–	–
V. racemosa Hutch & Dalziel TVNu 45 (w)	Zaire	–	+++	–	–

Table 1. continued.

Subgenera/sections/taxa†	Origin	K§	Q	I	A
Plectotropis					
V. kirki (Baker) Gilloet TVNu 364 (w)	Malawi	+++	+	–	–
V. kirki (Baker) Gilloet TVNu 865 (w)	Tanzania	+	+++	–	–
V. vexillata A. Richard var. *vexillata* TVNu 74 (w)	Rwanda	–	+++	–	–
V. vexillata A. Richard var. *macrosperma* TVNu 64 (w)	Australia	–	+++	–	–
V. vexillata A. Richard var. *vexillata* TVNu 72 (w)	Costa Rica	–	+++	–	–
Ceratotropis					
V. radiata (L.) R. Wilczek TVau 67 (w)	Indonesia	+	+++	(t)	(t)
V. radiata (L.) R. Wilczek TVau 58 (w)	Indonesia	+	+++	–	–

† c = cultivated; w = wild; M., M.&S. = Maréchal, Mascherpa et Stainier.

§ Relative amounts: K = kaempferol; Q = quercetin; I = isorhamnetin; A = apigenin; (+++) major flavonoid in the extract; (+) present; (t) present as trace; (–) absent.

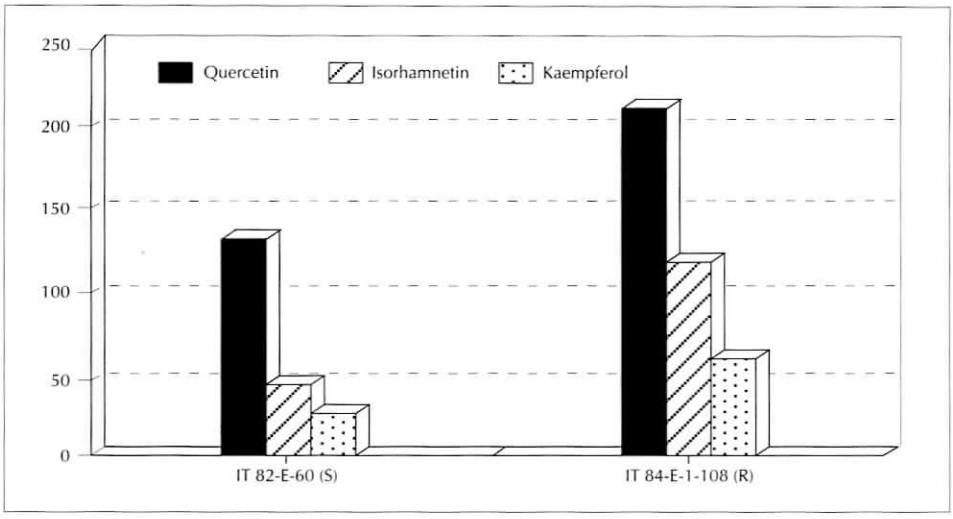

Figure 1. Flavonoid content (mg/100 g dry weight) in near-isogenic lines of *Vigna unguiculata* (L.) Walp. (R = resistant, S = susceptible).

analyzed lines were similar and showed 10 different glycosides; among them, 2 p-coumaroylglycosides of kaempferol and 5 of quercetin were found. Generally, wild species of *Vigna* lacked these more complex glycosides; only ssp. *dekindtiana* var. *dekindtiana* MG 112997 and *protracta* TVNu 965, ssp. *stenophylla* TVNu 714, and ssp. *tenuis* var. *tenuis* TVNu 661 contained one or two flavonol p-coumaroyl glycosides. These latter wild taxa, according to the classification scheme of Maréchal et al. (1978), revised by Ng and Maréchal (1985), are considered as subspecies of *V. unguiculata*, section *Catiang*. As regards the flavonoid glycoside content in cultivated lines of *V. unguiculata*, quantitative differences were found among the assayed lines. A positive relationship between resistance/susceptibility against aphids and flavonoid glycoside amount was also found. The resistant lines showed a flavonoid content higher than the susceptible ones. When the flavonoid aglycone content of two near-isogenic lines of *V. unguiculata* was considered (Fig. 1), the level in IT 84-E-1-108 (resistant) was twice that in IT 82-E-60 (susceptible).

There seems to be no reason to believe that resistance relationships found in domesticated plants do not occur also in wild ones. Thus, chemotaxonomic data on flavonoids in *Vigna* may also contribute to research in the field of plant ecology and crop protection. Besides these theoretical aspects of resistance/flavonoid relationships, additional information has been obtained from in vitro bioassays. In feeding experiments, 0.1 mM of some phenolics, in particular quercetin among the *Vigna* flavonoids, showed significant antifeedant activity on *Aphis fabae* (Scopoli) (Lattanzio et al. 1990; Lattanzio et al. 1992).

Wild species of Vigna

The wild species or subspecies of *Vigna* showed one, two, or rarely three flavonoid aglycones, and their flavonoid glycoside HPLC fingerprints were simpler than those of cultivated lines. The number of flavonoid glycosides ranged between two (*V. unguiculata* ssp. *dekindtiana* var. *pubescens* TVNu 110) and seven (*V. kirki* TVNu 865). None of the

Table 2. Flavonoid glycosides identified in some *Vigna* accessions.

Taxa	Flavonoid glycosides
V. unguiculata ssp. *dekindtiana* var. *dekindtiana* TVNu 413	Hyperoside (Quercetin-3-galactoside)
V. unguiculata ssp. *dekindtiana* var. *mensensis* TVNu 862	Robinin (Kaempferol-3-robinoside-7-rhamnoside)
V. unguiculata ssp. *dekindtiana* var. *protracta* TVNu 965	Robinin
V. unguiculata ssp. *stenophylla* TVNu 714	Kaempferol-3-rutinoside
V. ambacensis var. *ambacensis* TVNu 755	Kaempferol-3-rutinoside
V. gracilis var. *gracilis* TVNu 18	Robinin
V. heterophylla TVNu 19	Kaempferol-3-rutinoside, Isorhamnetin-3-rutinoside
V. marina var. *marina* TVNu 717	Robinin, Kaempferol-3-rutinoside
V. marina var. *oblonga* TVNu 1174	Isorhamnetin-3-rutinoside, Kaempferol-3-rutinoside
V. oblongifolia var. *oblongifolia* TVNu 135 and TVNu 37	Rutin (Quercetin-3-rutinoside)
V. racemosa TVNu 181	Rutin
V. racemosa TVNu 96 and TVNu 260	Rutin, Robinin
V. luteola TVNu 475	Rutin
V. luteola TVNu 172 and TVNu 905	Robinin
V. kirki TVNu 364 and TVNu 865	Hyperoside, Kaempferol-3-rutinoside
V. vexillata var. *vexillata* TVNu 72 and TVNu 74	Rutin
V. vexillata var. *macrosperma* TVNu 64	Rutin
V. radiata TVau 58 and TVau 67	Rutin, Kaempferol-3-rutinoside

flavonoid glycosides was found in the cultivated lines (Table 2). All compounds identified in the leaf extracts of *Vigna* were flavonol-3-O-glycosides, except for the less common 3,7-diglycosylation occurring in robinin (kaempferol-3-robinoside-7-rhamnoside). These glycosides contain the usual flavonoid sugar moieties such as glucose, rhamnose, and rutinose, besides the uncommon galactose and robinose (rhamnosylgalactose). The only compound detected in the three subgenera considered was Kaempferol-3-rutinosided.

With regard to the number of flavonoid aglycones in the leaf extracts (Table 1), there were three clear groups of species. The first group included species containing one aglycone: quercetin (*V. vexillata*, *V. oblongifolia*, *V. luteola* TVNu 475, and *V. racemosa* TVNu 45) or kaempferol (*V. luteola* TVNu 905 and *V. marina* TVNu 717). Other species showed only one aglycone present in detectable amounts plus traces of a second flavonoid aglycone (ssp. *dekindtiana*, var. *dekindtiana* TVNu 413, var. *mensensis* TVNu 862, and var. *pubescens* TVNu 110; ssp. *tenuis* var. *tenuis* TVNu 661; *V. gracilis* TVNu 18; *V. marina* TVNu 1174; and *V. luteola* TVNu 172). The second group included species containing two aglycones as well as, in some cases, traces of a third aglycone (ssp. *stenophylla* TVNu 714; *V. ambacensis* TVNu 755; *V. heterophylla* TVNu 19; *V. racemosa* TVNu 181; *V. racemosa* TVNu 260; *V. racemosa* TVNu 96; *V. kirki*; and *V. radiata*). Finally, the third group included subspecies of *V. unguiculata* (L.) Walp. (ssp. *unguiculata* and ssp. *dekindtiana* var. *dekindtiana* MG 112997 and var. *protracta* TVNu 965) belonging to section *Catiang*, containing three aglycones.

Two taxa of *Vigna* (ssp. *dekindtiana* var. *mensensis* TVNu 862 and *V. radiata* TVNu 67) showed traces of apigenin. In evolutionary terms, flavones are generally considered to be more advanced characters than flavonols by loss of 3-hydroxyl group (Harborne 1971; Williams et al. 1993; Zallocchi and Pomilio 1994). As only traces of apigenin were present, it would not be correct to speculate on the evolutionary significance of this flavone. However, further studies on ecogeographical distribution of apigenin in species of *Vigna* and other related genera suggests the process of diversification at work among and within legume species.

Intra- and interspecific relationships

Within the taxa analyzed, there was evidence of both intra- and interspecific chemical variation. Chemical analyses reflected the wide morphological variation in the genus *Vigna*. In addition, chromatographic data supported evidence for the existence of different flavonoid chemotypes in some of the species (*V. marina, V. unguiculata* ssp. *dekindtiana*, and *V. luteola),* which probably reflected the difference in geographic origin. There was extensive flavonoid glycoside variability encountered in this study.

The analyzed accessions of *V. marina* showed two completely different flavonoid patterns. Two chemotypes were identified, based upon a combination of aglycone structure and glycosylation pattern. One (TVNu 1174) contained two isorhamnetin glycosides and traces of two kaempferol glycosides, while *V. marina* TVNu 717 contained only kaempferol glycosides. Kaempferol-3-O-rutinoside was the only glycoside found in both accessions. These differences in flavonoid HPLC fingerprints are related to the wide morphological variation between the accessions. The two accessions of ssp. *dekindtiana* var. *dekindtiana* also showed flavonoid HPLC fingerprints as regards qualitative and quantitative aspects, MG 112997 being similar to the cultivated lines with regard to the number and the relative abundance of flavonoid aglycones, and the presence of one p-coumaroyl glycoside of quercetin. Otherwise, TVNu 413 contained only three quercetin glycosides. *V. luteola* accessions also showed two different chemotypes. The accession TVNu 475 contained only quercetin (rutin and a second quercetin glycoside), while the other two accessions, the kaempferol chemotypes TVNu 172 and TVNu 905, were similar qualitatively with some quantitative differences in their flavonoid glycoside pattern; both contained robinin.

No chemotypes were distinguishable among the accessions of *V. racemosa, V. vexillata, V. oblongifolia, V. kirki,* and *V. radiata*, collected from different geographical zones.

As regards interspecific relationships, species of sections *Vigna*, *Plectotropis*, and *Ceratotropis* did not show flavonoid glycosides similar to those in cultivated lines of *Vigna*. In contrast, some relationships have been found between cultivated lines and wild species of the section *Catiang*. The flavonoid HPLC fingerprint of ssp. *dekindtiana* var. *dekindtiana* MG 112997 and ssp. *dekindtiana* var. *protracta* TVNu 965 was very similar to the cultivated lines, having four flavonoid glycosides, including the rare acyl glycosides. The occurrence of similar flavonoid patterns in morphologically distinct taxa suggests they had a common ancestor and that climate and habitat changes could have caused them to adapt morphologically, while retaining, in part, their original leaf flavonoid pattern (Panella et al. 1993; Williams et al. 1993). Minor relationships were found between cultivated lines and the other subspecies of the section *Catiang*: ssp. *stenophylla* TVNu 714 and ssp. *tenuis* TVNu 661 had two glycosides, while ssp. *dekindtiana* var. *mensensis* TVNu 862 and ssp.

dekindtiana var. *pubescens* TVNu 110 had only one glycoside in common with ssp. *unguiculata*.

In the section *Vigna*, some relationships were observed among *V. ambacensis* TVNu 755, *V. gracilis* TVNu 18, *V. marina* TVNu 717, and *V. racemosa* TVNu 96 (kaempferol chemotypes of this section based upon a combination of aglycone class [Table 1] and glycosylation pattern, because two kaempferol glycosides in common were found in these species). The quercetin chemotypes of section *Vigna*—*V. luteola* TVNu 475, *V. oblongifolia* TVNu 37, and *V. oblongifolia* TVNu 135—also showed a great similarity to one another. This agrees with the results obtained by Vaillancourt and Weeden (1993) using molecular markers. Overall, the isorhamnetin chemotypes of this section—*V. marina* TVNu 1174 and *V. heterophylla* TVNu 19, both containing kaempferol-3-rutinoside and isorhamnetin-3-rutinoside—were remarkably similar to one another. The presence of 3'-O-methylation in the B-ring of quercetin (isorhamnetin) could be considered a fairly advanced character, absent in other wild species containing quercetin and/or kaempferol (Zallocchi and Pomilio 1994). The common flavonoids in this section seem to be robinin (*V. gracilis, V. marina, V. racemosa*, and *V. luteola*), rutin (*V. oblongifolia, V. racemosa*, and *V. luteola*), kaempferol-3-rutinoside (*V. ambacensis, V. heterophylla*, and *V. marina*), and an unidentified (tR = 29.56 min) kaempferol glycoside (*V. ambacensis, V. gracilis, V. marina, V. racemosa*, and *V. luteola*). Rutin and kaempferol-3-rutinoside were also found in *V. radiata*, section *Ceratotropis*. Finally, in the section *Plectotropis, V. vexillata* containing rutin, and *V. kirki* containing kaempferol-3-rutinoside and hyperoside, represent two different flavonoid chemotypes according to their aglycone structure and glycosylation pattern.

In conclusion, flavonoid HPLC fingerprints together with other biochemical markers and/or morphological data can provide useful characters for defining species in the *Vigna* genus. From Table 1, it is evident that *Vigna* species produce essentially flavonol structures that are usually 3-O-glycosides. The most characteristic feature of these compounds in *Vigna* is the presence of flavonoid p-coumaroyl glycosides in cultivated lines, while the wild species are generally devoid of these substances, with the exception of four wild species in the section *Catiang*, and are all classified as subspecies of *V. unguiculata*. A greater variability in flavonoid aglycone class and glycosylation pattern occurs in cultivars of *V. unguiculata* ssp. *unguiculata* compared to the wild species. This observation seems to confirm that cultivation and/or domestication may cause or increase species diversification. The large differences between cowpea and wild species of *Vigna* may indicate that the cowpea has been isolated from other species for a very long time, and thus accumulated a large genetic diversity. This ancient genetic divergence may, in part, explain the lack of success in hybridization between cowpea and other species of this genus (Fatokun 1991; Vaillancourt and Weeden 1992).

Furthermore, as regards the role of endogenous flavonoids in the resistance mechanism against aphids, it has been frequently pointed out that these compounds which taxonomists use to separate species could hardly have had enough adaptive value for survival through natural selection. In fact, when the resistance characteristics to aphids in different accessions of the same species of *Vigna* have been considered, it became evident that quercetin chemotypes show a higher level of resistance compared to the kaempferol ones. These results provide useful information to further explore the gene expression of the resistance factors.

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