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Constitutive Flavonoids and Induced Isoflavonoids as Taxonomic Markers in the Genus *Vigna*

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Key Word Index—*Vigna*; Leguminosae; phytoalexins; isoflavonoids; flavonol glycosides; glycosylflavones.

Abstract—A survey of phytoalexin production in eight species and three subspecies of *Vigna* showed that the response was similar in all taxa. Dalbergioidin, kievitone and phaseollidin were produced in the hypocotyl, epicotyl or seed, while only the first two compounds were formed in the root. Phaseollin was detected in leaf tissue of *V. unguiculata* and *V. radiata* by HPLC, but it was not present in tissue diffusates. A survey of constitutive flavonoids in leaf, stem, flower and seed coat showed that all but three species (*V. mungo*, *V. angularis* and *V. umbellata*) could be distinguished from each other by this means. *Vigna radiata* and *V. trilobata* differed from the other taxa in having quercetin glycosides in addition to the kaempferol glycosides more generally present. *Vigna unguiculata* was distinct in being depauperate in flavonoids. Three kaempferol 3,7-diglycosides were identified, the 3-robinobioside-7-rhamnoside, the 3-robinobioside-7-glucoside and the 3-glucoside-7-rhamnoside and these were restricted to the leaves. By contrast, the stems contained primarily kaempferol 3-rutinoside, the flowers kaempferol 3-sophoroside and the seed coats vitexin and isovitexin.

Introduction

In previous chemotaxonomic investigations of plants of the Leguminosae in this laboratory, constitutive flavonoids (Webb and Harborne, 1991) constitutive isoflavonoids (Harborne, 1969) and isoflavonoids induced by fungal inoculation (Robeson and Harborne, 1980) have been shown to be useful taxonomic markers at the generic level. Thus, a survey of leaf flavonoids in the genus *Vicia* showed some interesting correlations between the distribution of flavones and flavonols and sectional classification (Webb and Harborne, 1991). Likewise, an investigation of isoflavonoid phytoalexin induction in the genus *Lathyrus* showed that two morphologically isolated species, *L. hirsutus* and *L. odoratus*, were also distinctive in the phytoalexins formed after fungal inoculation (Robeson and Harborne, 1980). But in no previous investigation have both constitutive and induced constituents been compared as taxonomic markers within the same plant group. The present study of cultivated species of *Vigna* was initiated in order to make such a comparison.

The genus *Vigna* contains several valuable crop species, notably the mungbean *V. radiata* and the cowpea *V. unguiculata*. The taxonomy of the genus has been very confused because of the difficulty of its delimitation from *Phaseolus*. Indeed, in the past, several cultivated *Vigna* species have been included with the French bean *Phaseolus vulgaris* under *Phaseolus*. In most modern treatments, *Vigna* is recognized as containing both the mungbean and cowpea and consists of about 160 species, divided into seven subgenera and 11 sections (Marechal *et al.*, 1978). The advantage of studying the cultivated species of *Vigna* is their availability as authenticated seed and the fact that some information is already in the literature on both constitutive and induced metabolites. Earlier work on the leaf flavonoids has shown that the kaempferol 3,7-glycoside, robinin, occurs in *V. angularis* and *V. mungo*, while rutin is present in *V. radiata*; three other kaempferol glycosides have also been reported from these species (Ishikura *et al.*, 1981).

Previous studies of phytoalexin induction have revealed that some 16 isoflavonoid

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structures may be formed in *Vigna*, as well as the benzofuran vignafuran reported from *V. unguiculata* (Preston *et al.*, 1975). The picture, however, is a confusing one and some structures such as genistein and 2'-hydroxygenistein are probably biosynthetic intermediates of the isoflavanone kievitone and the pterocarpan phaseollidin, rather than phytoalexins *per se*. Other compounds reported are not true phytoalexins, because they have only been detected as stress metabolites (Abe *et al.*, 1987). The only comparative study of phytoalexin induction in different species of *Vigna* was carried out by Ingham (1990) while our studies were in progress. He examined five species as part of a broad survey of 76 plants of the tribe Phaseoleae and reported dalbergioidin, kievitone, phaseollidin and demethylmedicarpin after leaf inoculation with *Helminthosporium carbonum*. As we describe below, the leaf in *Vigna* responds weakly to drop diffusate techniques and other tissues give a much more reliable response.

We therefore describe here a comparative investigation of phytoalexin induction in 53 accessions of eight species and three subspecies of *Vigna*, using seed, hypocotyl, epicotyl and root as well as leaf. We have also compared the fungitoxicities of the major phytoalexins against 16 pathogenic fungal species. Additionally, we record the flavonoid patterns that are present in stem, leaf, flower, seed and root of the same plants.

Materials and Methods

Plant material. Most seed samples were supplied by international institutes and were of known provenance (see Acknowledgements); a few accessions were of Botanic Garden origin or were obtained by purchase from local stores. All seed accessions were grown to mature plants in a warm glasshouse and taxonomic identifications were checked by morphological measurements (e.g. pod length), seed size and colour, leaf shape and other key characteristics and by comparison with herbarium specimens.

Phytoalexin identifications. These were carried out by standard procedures, after separation and purification by TLC on thick silica gel plates. They were based on UV spectral (and shift) measurements, R_f and *RR*, comparisons, EI-MS and fragmentation patterns, and by ^{1}H NMR spectroscopy. Standard markers of phaseollin and phaseollin isoflavan were obtained from a large scale phytoalexin induction experiment with seed of *Phaseolus vulgaris*.

Flavonoid identifications. These were carried out by standard procedures, after separation and purification by paper chromatography in several solvent systems. They were based on UV spectral (and shift) measurements, R_f comparisons and hydrolysis to yield aglycone and sugars. Sugars were identified by co-chromatography against markers in four solvent systems, including toluene–pyridine–acetic acid–water (4:1:1:3) which gives a clear separation of glucose and galactose. Flavonol 3,7-glycosides were further characterized by partial acid and by enzymic hydrolysis in order to locate the 3- and the 7-sugars unambiguously. In nearly all cases, co-chromatography with authentic markers in at least four solvent systems was carried out to confirm identifications.

Results

Phytoalexin induction

Preliminary experiments showed that the standard drop diffusate technique (Ingham, 1990) with leaves of all eight *Vigna* species tested did not provide a satisfactory procedure for a phytoalexin survey. Although the leaves responded to inoculation with spores of *Botrytis cinerea, Cladosporium herbarum* and other fungi (see Table 3) and with dilute copper sulphate solutions, the phytoalexins remained mainly within the leaf and only trace amounts were released into the droplets. While it was possible to extract the leaves at the end of the experiment, the overlapping presence of many constitutive phenolic constituents made it difficult to separately detect the phytoalexins on TLC plates. Attention was therefore turned to the seed slicing technique of Keen (1975) whereby endogenous microflora are allowed to induce phytoalexin formation. Diffusate experiments were also set up on the hypocotyl or epicotyl and the root of young seedlings.

The results of surveying hypocotyl or epicotyl tissue of the various accessions of the eight *Vigna* species are shown in Table 1. It is apparent that, with few exceptions, all

TABLE 1. PHYTOALEXINS FROM HYPOCOTYL OR EPICOTYL TISSUE OF *VIGNA* SPECIES†

Plant species*‡ and accession number	Presence/absence of			
	Dalbergioidin	Kievitone	Cpd-1	Phaseollidin
V. aconitifolia (Jacq.) Marechal				
*MoA1	+	-	+	+
P-32	-	-	-	
PG-1	-	+	+	
*509	-	+		-
V. angularis (Willd.) Ohwi & Ohashi				
*AA1	+	+	+	-
*5125	+	+	+	-
S-5	+	+	+	+
*Ahevi 936	+	+	+	+
V. mungo (L.) Hepper				
*BA1	+	+	+	+
*TVm1	+	+	-	-
TVm2	+	+	+	-
*T-9	+	+		
MI-1	+	+	+	-
3118	+	+	+	+
*3119	+	+		+
3120	-	-		+
V. radiata (L.) Wilczek				
*MA1	+	+	+	+
*T-51	+	-	+	+
T-77	+	+		-
M-24	+	-		
TVau 5	+	+	+	-
TVau 6	+	+	+	+
TVau 12	+	-	+	+
*V2494	-	+		+
V2810	+	+		+
V. subterranea (L.) Verdc.				
Rober 3	+	+		-
*Ankp 24	+	-		+
Ankpol	-	+		+
*Ghana Tali	+	-		+
V. trilobata (L.) Verdc.				
*N10251		+		
N10258		-		
V. umbellata (Thunb.) Ohwi & Ohashi				
*436	-	+	+	-
*4022	+	+	+	
*4042	+	-	+	
4074	+	+	+	-
TVum 1	+	+		
V. unguiculata (L.) Verdc.				
ssp. *unguiculata*				
*CA1	-	+	+	+
*362	+	+	+	+
971	+	+		-
*Arlington	-	+	+	+
ssp. *cylindrica*				
*360	-	-	+	+
2078	+	+	-	-
392	+	+		-
359	+	+	-	-
*Hawarimae	+	+	-	+

TABLE 1—*CONTINUED*

Plant species*‡ and accession number	Presence/absence of Dalbergioidin	Kievitone	Cpd-1	Phaseollidin
ssp. *sesquipedalis*				
*361	+	+	+	+
*Polonmae	+	+	+	+
*Bushitawo	+	+	+	+

†Epicotyl tissue in the case of *V. angularis*, *V. umbellata* and *V. subterranea* hypocotyl tissue in the other five species.

‡All taxa were induced with copper sulphate solution; those with an asterisk were additionally treated with *Cladosporium herbarum*.

taxa respond identically with the production of four phytoalexins, dalbergioidin, kievitone, phaseollidin and one unidentified substance (compound **1**). Their antifungal properties were confirmed by bioassay and by more detailed fungitoxicity tests described below. Similar results were obtained in experiments with seed tissue, while a survey of root tissues showed that only dalbergioidin and kievitone were formed in detectable amounts by the roots (Table 2). Phaseollin and phaseollin isoflavan, which are two phytoalexins of *Phaseolus vulgaris*, were run as markers in many experiments but neither could be detected in either control or induced diffusates.

The three phytoalexins, dalbergioidin, kievitone and phaseollidin, were readily identified by UV, MS and NMR measurements and comparison with literature data (see Materials and Methods). The fourth phytoalexin, compound **1**, had a UV spectrum typical of an isoflavone and a molecular weight (270 M_r) identical to genistein, but it could not be further identified. It was not demethylmedicarpin (256 M_r) reported by Ingham (1990) as a trace component in leaf diffusates of *Vigna*. Otherwise, our results with other tissues are closely similar to those of Ingham (1990) with leaves. He was unable to detect phaseollidin in *V. angularis* and *V. mungo*, whereas we found it in these two species as well as elsewhere (Table 1).

Because of the poor response of the leaf tissue to the drop diffusate technique and subsequent TLC analysis (see above), we carried out preliminary HPLC analyses on leaf diffusates and leaf extracts. As a result, we were able to detect phaseollin as a phytoalexin in *V. unguiculata* by co-HPLC, confirming a much earlier observation from viral induction of Bailey (1973). Phaseollin was similarly detected in *V. radiata* for the first time. However, it was not detectable in diffusates but only from leaf extracts, so that its absence from our main experiments must be due to its failure to be released into the diffusate. Our HPLC analyses also indicated that dalbergioidin may not be a true phytoalexin in the leaf, since it could be detected in control experiments as a constitutive component.

TABLE 2. PHYTOALEXIN RESPONSE IN DIFFERENT TISSUES OF EIGHT *VIGNA* SPECIES

Part of plant investigated*	Phytoalexins detected§ Dalbergioidin	Kievitone	Compound **1**	Phaseollidin
Seeds†	+	+	+	+
Hypocotyls or epicotyls‡	+	+	+	+
Roots‡	+	+	+	+

*Leaves responded positively, but it was too difficult to detect phytoalexins by TLC; phaseollin was detected by HPLC in leaf tissue of *Vigna anguiculata* and *V. radiata*.

†Seeds were challenged (a) with their own microflora; and (b) with *Botrytis cinerea*.

‡Challenged with (a) copper sulphate solution; (b) *Cladosporium herbarum*; and (c) *Botrytis cinerea*.

§Daidzein was detected in many experiments, but it was also found in lesser amounts in the control.

The effects of three of the *Vigna* phytoalexins on mycelial growth and spore germination in selected pathogenic fungi have been compared (Tables 3, 4). It is interesting that kievitone is more fungitoxic than dalbergioidin or phaseollin in its effect on mycelial growth, while there is little difference between the three compounds as inhibitors of spore germination. Kievitone is also quite variable in its effects on the mycelial growth of different fungi; *Alternaria tenuis* (kievitone ED_{50} 8 μg ml^{-1}) is much more sensitive than is *Colletotrichum lindemuthianum* (94 μg ml^{-1}). Similar variations, however, have already been reported before in relative fungitoxicities of isoflavonoid phytoalexins (Harborne and Ingham, 1978).

Constitutive flavonoids

The results of surveying eight *Vigna* species for the leaf flavonoids (Table 5) considerably extend our knowledge of the chemistry of the genus; only two species, *V. mungo* and *V. radiata*, have been examined in detail before. Four kaempferol glycosides, the 3-glucoside, 3-glucoside-7-rhamnoside, 3-robinobioside-7-glucoside and 7-glucoside are recorded in *Vigna* for the first time. All nine flavonol glucosides (Table 5) were identified by standard procedures (see Materials and Methods) and by co-chromatography with authentic markers.

TABLE 3. EFFECT OF KIEVITONE, DALBERGIOIDIN AND PHASEOLLIN ON THE MYCELIAL GROWTH OF SELECTED FUNGI

	ED_{50} (μg ml^{-1})		
Fungus	Kievitone	Dalbergioidin	Phaseollin
Aspergillus niger	72	84	76
Botrytis cinerea	48	40	70
B. fabae	81	83	31
Colletotrichum lindemuthianum	94	91	83
Fusarium oxysporum	72	63	ND
Alternaria brassicola	13	16	ND
A. tenuis	8	15	70
Ascochyta phaseolorum	13	19	ND
Cercospora canescens	28	33	ND
Cladosporium herbarum	17	21	84
Fusarium solani	5	10	ND
F. culonarum	5	8	ND
Helminthosporium sp.	15	27	67
Phoma betae	8	8	79
P. foreata	14	15	ND
P. eupyrena	20	28	ND

ND = not determined.

TABLE 4. EFFECT OF KIEVITONE, DALBERGIOIDIN AND PHASEOLLIN ON THE SPORE GERMINATION OF SOME FUNGI

	ED_{50} (μg ml^{-1})		
Fungus	Kievitone	Dalbergioidin	Phaseollin
Aspergillus niger	33	23	ND
Botrytis cinerea	41	41	33
B. fabae	33	30	ND
Colletotrichum lindemuthianum	37	40	35
Alternaria brassicola	52	48	ND
A. tenuis	37	32	41
Cladosporium herbarum	27	31	37
Fusarium solani	48	43	ND
Helminthosporium sp.	38	31	53

ND = not determined.

TABLE 5. FLAVONOL GLYCOSIDES IDENTIFIED IN LEAVES OF *VIGNA* SPECIES

Plant species and accession number	Quercetin glycosides		Kaempferol glycosides						
	1	2	3	4	5	6	7	8	9
V. aconitifolia									
*MoA1				−	+			+	−
502				−		·		−	+
512				+		−		·	−
PG-1				−	·			−	·
V. angularis									
AA1			−	·	+	−			
5125			+	−	·				
5127			−	+	−				
Ahevi 936			·	+	−	+			
V. mungo									
BA1			·	−	+	·			
TVm1			+	−	−	·			
T-9			−	+	−	−			
3118			−	·	·				
V. radiata									
MA1	·	−		−		−			
T-51	−	+		·		−			
M-24	−	·		+		−			
TVau 5	+	·		·	·				
V. subterranea									
Roger 3				·	+	−	+		
Ankp 24				−	·	·	+		
Ankpol				+	·	·	·		
Ghana Tali				·	−	+	−		
V. trilobata									
N10251	·	−		−		−			
N10258	·	+		·		−			
V. umbellata									
436			+	·	·				
4022			+	·	+				
4042			−	−	+				
TVum 1			−	·	·				
V. unguiculata ssp. *unguiculata*									
CA1				−	+				
362				−	−				
ssp. *cylindrica*									
360				−	+				
Hawarimae				−	+				
ssp. *sesquipedalis*									
361				+	·				
Bushitawo				−	+				

1 = Isoquercitrin; 2 = rutin; 3 = robinin; 4 = kaempferol 3-rutinoside; 5 = kaempferol 7-rhamnoside; 6 = kaempferol 3-glucoside; 7 = kaempferol 3-glucoside-7-rhamnoside; 8 = kaempferol 3-robinobioside 7-glucoside; 9 = kaempferol 7-glucoside.

While all eight species have kaempferol glycosides, only *V. radiata* and *V. trilobata* contain the quercetin glycosides, rutin and isoquercitrin, as well. Again, *V. aconitifolia* is distinct in producing kaempferol 3-robinobioside-7-glucoside and kaempferol 7-glucoside, while *V. subterranea* differs from the other species in synthesizing kaempferol-3-glucoside-7-rhamnoside. By contrast, *V. unguiculata* differs in being

TABLE 6. FLAVONOL GLYCOSIDES IDENTIFIED IN FLOWERS AND STEMS OF *VIGNA* SPECIES

Plant species and accession number	Flavonol glycosides of*						
	Flowers				Stems		
	1	2	3	4	1	4	5
V. aconitifolia							
MoA1			+	·		−	−
502			+	−		+	·
PG-1			−	+		+	·
V. angularis							
AA1			+	−		+	+
V. mungo							
BA1			+	−		−	+
TVm1			+	−		−	+
T-9			+	−		−	+
3118			+	−		−	
V. radiata							
MA1	+	−	+	+	+	·	+
T-51	+	+	−	+	+	−	−
M-24	+	+	−	+	+	+	−
TVau 5	−	+	−	−	−	+	−
V. subterranea							
Roger 3			+	−		·	+
Ankp 24			+	−		·	+
V. trilobata							
N10251	+		+	+	+	·	−
N10258	−		−	+	−	+	−
V. umbellata							
436			·	+		+	−
4022			−	+		+	
4042			+	−		+	
TVum 1			+	·		·	−
V. unguiculata							
ssp. *unguiculata*							
CA1			−			−	
362			−			−	
ssp. *cylindrica*							
360			+			−	
Hawarimae			+			+	
ssp. *sesquipedalis*							
361			+			+	
Bushitawo			+			·	

1 = Isoquercitrin; 2 = rutin; 3 = kaempferol 3-sophoroside; 4 = kaempferol 3-glucoside; 5 = kaempferol 3-rutinoside.

depauperate in flavonoids, having only two simple glycosides, the 3-rutinoside and the 7-rhamnoside (Table 5).

The results of surveying stems and flowers for flavonoids (Table 6) show that these tissues have fewer glycosides than the leaves. In the flowers, the dominant kaempferol 3-rutinoside of leaves and stems is unexpectedly replaced by the corresponding 3-sophoroside. The restriction of quercetin glycosides to *V. radiata* and *V. trilobata* recorded in the leaf (Table 5) also extends to the stem and floral tissue (Table 6).

A survey of seed coats in *Vigna* showed that none of the flavonol glycosides, so abundantly present elsewhere in the plant, was present. Instead, the two *C*-glycosyl-flavones, vitexin and isovitexin, reported by Ishikura *et al.* (1981) in *V. mungo* and *V.*

radiata, were found to occur in all taxa, except *V. unguiculata*. This striking absence of flavonol glycosides from the seed coat of *Vigna* species contrasts with the situation in *Phaseolus vulgaris*, where flavonol glycosides occur in flower, leaf and seed coat (Harborne, 1971). The seed coats of all species, including *V. unguiculata*, additionally contained procyanidin and prodelphinidin.

Finally, the roots were surveyed, but none of the flavonoids so far reported were present. Instead, the roots were universally shown to have the two isoflavones, genistein and daidzein, in glycosidic combination.

Discussion

The results of this survey of *Vigna* for phytoalexins have shown that the leaf drop diffusate technique, that has been so widely applied for detecting these induced chemicals in the Leguminosae (e.g. Ingham, 1990), is not necessarily the most reliable procedure in every case. Both epicotyl or hypocotyl tissue in one set of experiments and sliced seed in another gave excellent results throughout the 53 accessions tested. The results with eight *Vigna* species confirm the view that within the group of cultivated taxa of this genus, the phytoalexin response is very similar. Dalbergioidin, kievitone and phaseollidin are the major compounds formed. Whether phaseollin, which is closely related (by ring closure of the isopentenyl substituent) to phaseollidin, is also a phytoalexin in all species remains for further investigation. We have confirmed that it is formed in the leaf of *V. unguiculata* and *V. radiata*. Of these two species, *V. unguiculata* does seem to be different from the rest surveyed in its ability to produce additional phytoalexins, as recorded in earlier experiments (e.g. Preston *et al.*, 1975). How far the pattern of phytoalexin synthesis in *Vigna* differs from *Phaseolus* remains for the future, but we could not detect phaseollin isoflavan, a phytoalexin of *P. vulgaris*, in any of our *Vigna* accessions.

The results of surveying the constitutive flavonoids in eight *Vigna* species reveal that five have species-specific patterns. Three species are the same: *V. mungo*, *V. umbellata* and *V. angularis*; and these are all in the same subgenus *Ceratotropis*. It may be noted that *V. unguiculata*, in a separate section *Catjang* of the subgenus *Vigna*, is most different from any other species in its flavonoids. It has a simple pattern of flavonol glycosides throughout the plant.

From these data, it would appear that the constitutive flavonoids offer more chemical characters for infrageneric classification than phytoalexin induction. These results, together with those from other legume surveys, might suggest that constitutive chemicals are more useful at sectional and generic level, while induced chemicals vary more above the generic level. Nevertheless, there is still a considerable element of unpredictability in chemotaxonomic investigations of legume plants, and this should not discourage future workers from examining both types of chemical character at any level.

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