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PARA-AMINOPHENYLALANINE IN *VIGNA*: POSSIBLE TAXONOMIC AND ECOLOGICAL SIGNIFICANCE AS A SEED DEFENCE AGAINST BRUCHIDS

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Key Word Index—*Vigna*; Leguminosae; *Callosobruchus maculatus*; *Zabrotes subfasciatus*; Bruchidae; bruchid; non-protein amino acid; *p*-aminophenylalanine; host–plant resistance; chemotaxonomy.

Abstract—Seeds of eight wild species and varieties from *Vigna* subgenera *Plectotropis* and *Vigna* were screened for the non-protein amino acid *p*-aminophenylalanine (PAPA), previously reported to have a restricted taxonomic distribution and to be a growth inhibitor of *Escherichia coli*. The compound was detected in five wild species and its distribution found to have taxonomic value for assessing members of *Plectotropis* and intrageneric links with other taxa. Although quantitative variation in PAPA was detected between species and also between varieties of *V. vexillata*, toxicity tests confirmed that the levels detected in all accessions were sufficient for PAPA to be an important component of resistance against two important pest bruchids of *Vigna* and *Phaseolus* crops. The taxonomic and ecological significance of these findings is discussed.

INTRODUCTION

The genus *Vigna* (Leguminosae) contains about 90 species, of which several are important crops (e.g. cowpea, *V. unguiculata*). Although frequently revised by taxonomists, the genus has most recently been divided into two New World (*Sigmoidotropis*, *Lasiospron*) and five Old World (*Plectotropis*, *Vigna*, *Ceratotropis*, *Haydonia*, *Macrorhyncha*) subgenera [1]. Intrageneric relationships within *Vigna* and also the intergeneric limits and links with the New World genus *Phaseolus* pose several taxonomic problems. One such problem of particular interest is the relationship of the small Old World subgenus *Plectotropis* (seven species) with other subgenera in *Vigna*. According to Maréchal *et al.* [1, 2], this group not only links the two large Old World subgenera *Vigna* and *Ceratotropis*, but also forms a bridge linking these to the New World subgenus *Sigmoidotropis* (Fig. 1). In two previous studies of seed chemistry, one covering 40/90 *Vigna* spp. and 16/31 *Phaseolus* spp. [3] and the other covering a large but undefined number of Phaseolinae [4], the non-protein amino acid analogue of phenylalanine, *p*-aminophenylalanine (PAPA, **1**), previously reported as a growth inhibitor of *E. coli* [5], was only detected in subgenera *Plectotropis* (three species) and *Vigna* (one species) [4, 6]. The acquisition of three further taxa from *Plectotropis* has enabled us to re-examine the taxonomic significance of PAPA as proposed by Dardenne [6] in defining membership of *Plectotropis* and links with other subgenera. However, we were still only able to obtain seed of five of the seven species within this group (Table 1), omitting the rare species *V. davyi* and *V. hundtii* but including three out of six varieties of *V. vexillata*, a species previously noted to vary in PAPA seed concentration between different seed collections [6]. We also included *V. angivensis* from subgenus *Vigna* [1] to confirm previous reports [3, 6] that this single species outside the established members of *Plectotropis* also contains PAPA.

Several non-protein amino acids found in legume seeds, including the structurally related compound L-3,4-dihydroxyphenylalanine (L-DOPA), have been shown to have insecticidal activity against a range of seed predators [8, 9]. We have tested the possibility that PAPA may contribute to the chemical defences of wild *Vigna* seeds against predation by larvae of two generalist species of seed-eating bruchid beetles, which are major pests of several crops from the *Vigna*/*Phaseolus* complex [10].

RESULTS AND DISCUSSION

Accessions of five of the seven *Vigna* species conventionally placed in *Plectotropis* were available in sufficient quantity for a semi-quantitative screen of PAPA seed content (Table 1). PAPA was detected in all species (including previously unscreened taxa *V. vexillata* var. *macrosperma*, *V. lobatifolia*, *V. longissima*) except *V. kirkii*, confirming a preliminary report of its absence in the latter species [6]. The inclusion of *V. kirkii* in *Plectotropis* is controversial since Maréchal considers it to be in an intermediate position between *Plectotropis* and *Vigna* but included it in the former due to its flower asymmetry [1], whilst Verdcourt transferred it into the latter group [7]. The absence of PAPA suggests that *V. kirkii* may be intermediate between the two groups since its flower morphology links it with *Plectotropis* but its seed chemistry places it outside the group. The chemical screen also confirmed the reported occurrence of PAPA in a single

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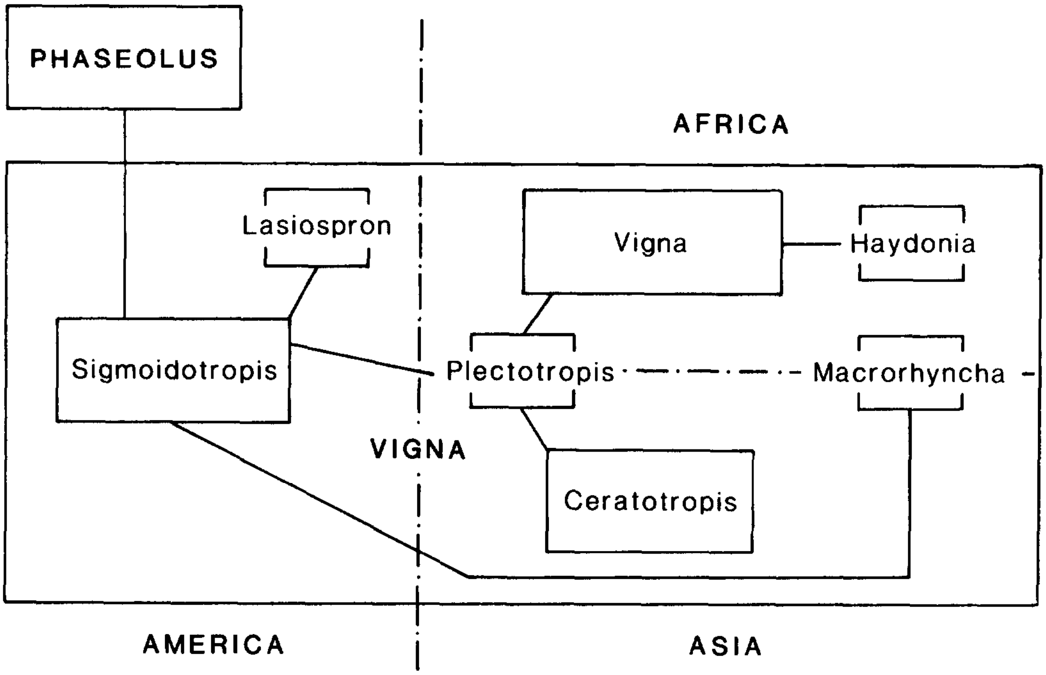
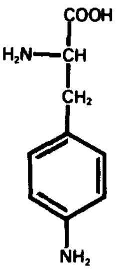


Fig. 1. Taxonomic relationships between subgenera and genera in the *Vigna/Phaseolus* complex (after ref. [1]).



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species from subgenus *Vigna*, *V. angivensis* [6]. This endemic of Madagascar, although placed by Maréchal with the African *Vigna* group, again shows morphological characteristics suggesting strong links with *Plectotropis* [1]. PAPA was not detected in members of the small taxonomically linked New World group subgenus *Sigmoidotropis* section *Peduncularis* nor in members of the linked Old World group, subgenus *Ceratotropis* (unpublished results), possibly indicating closer links between *Plectotropis* and *Vigna* than other groups linked in Fig. 1.

Dardenne [6] observed that levels of PAPA in seed of *V. vexillata* varied with accession and type of cultivation between 0.1 and 1.3 % fresh weight. Quantitative determination of PAPA in seed of several accessions of *V. vexillata* (including vars. *vexillata*, *angustifolia*, *macrosperma*) showed differences within the range previously detected (Table 2). This may reflect growth conditions of each collection but could also indicate the existence of genetically determined intraspecific differences. Quantitative differences in seed defence chemicals, which need only be small if the compound is sufficiently toxic, may well be important in reducing selection pressure for seed predators to counter-adapt by developing specialized detoxification mechanisms [11].

Seeds of all *Vigna* accessions listed in Table 3 were screened for resistance to two generalist species of bruchid beetle, *Callosobruchus maculatus* (of Old World origin) and *Zabrotes subfasciatus* (of New World origin), both major pests of crops in the *Vigna/Phaseolus* complex. No live adults of *Z. subfasciatus* emerged from any seed assayed, although beetles laid viable eggs on all accessions except *V. kirkii* (due to the small size of seed or seed surface oviposition inhibitors).

Callosobruchus maculatus was able to lay eggs on and complete its life cycle in seed of *V. kirkii* (PAPA not detected), on which one adult emerged per seed (equivalent to 85 adults per g seed, i.e. highly susceptible on the scale published previously [10]). However, larvae of *C. maculatus* were not able to develop in seeds of any PAPA positive accessions. These results suggested that PAPA may be a component of seed resistance in wild *Vigna* species accumulating the compound. It was also noteworthy that the species *V. kirkii*, a disputed member of *Plectotropis* on morphological and chemical grounds, was also conspicuously lacking in resistance to *C. maculatus* compared to other established members of *Plectotropis*. Insect resistance characteristics of plants may provide useful taxonomic information, as previously described by Janzen and others [11, 12].

To investigate further the toxicity of PAPA to both pest bruchids, the compound was incorporated into artificial seeds at a range of naturally occurring concentrations, up to a level causing 100 % larvel mortality (Tables 4 and 5). PAPA clearly had a detrimental effect on the larval survival and development of both bruchids in a dose-dependent manner, but *Z. subfasciatus* (lethal dose 0.3 %) was noticeably more susceptible than *C. maculatus* (lethal

Table 1. Results of semi-quantitative screen of *Vigna* subgenus *Plectotropis* seed for PAPA, using high voltage electrophoresis

Species	S/G*	Documentation†	PAPA (Semi-quantitative)‡	Previous investigations§
V. vexillata (L.) A. Rich.	P	TVNu84 IITA	+	
V. vexillata	P	TVNu72 IITA	++	D76
V. vexillata	P	CPI 17457 CSIRO	+	
V. vexillata var. *vexillata*	P	N.I. 460 Gembloux	+	D76
V. vexillata var. *angustifolia* (Schum. & Thonnn.) Bak.	P	N.I. 552 Gembloux	+	D76
V. vexillata var. *macrosperma* Maréchal *et al.*	P	N.I. 339 Gembloux	+	n.p.i.
V. kirkii (Bak.) Gillet	P or V	N.I. 448 Gembloux	n.d.	D76
V. lobatifolia Bak.	P	N.I. 546 Gembloux	++	n.p.i.
V. nuda N.E.Br.	P	Mutimushii 995, R.B.G.K.	+	D76
V. longissima Hutch.	P	Brummit 13891 R.B.G.K.	+	n.p.i.
V. angivensis Bak.	V	N.I. 378 Gembloux	+	D76

*S/G, Subgenus [1, 7]. P = *Plectotropis*, V = *Vigna*.

†IITA, International Institute of Tropical Agriculture; CSIRO, Commonwealth Scientific and Industrial Research Organization; Gembloux, Faculté des Sciences Agronomiques de Gembloux, base collection of wild and botanical forms of Phaseoleae; R.B.G.K, Royal Botanic Gardens, Kew.

‡n.d., not detected (< 0.01%); +, low (0.01–0.1%); ++, moderate (0.1–0.3%); +++, high (> 0.3%).

§D76, Dardenne, 1976 (confirmation); n.p.i., not previously investigated.

Table 2. Quantitative estimation of PAPA in seeds of *Vigna* spp. from subgenus *Plectotropis*

Species	Variety	Accession	% PAPA (fr. wt)
V. vexillata	—	TVNu84	0.09
V. vexillata	—	TVNu72	0.29
V. vexillata	—	CPI 17457	0.10
V. vexillata	*vexillata*	N.I. 460	0.08
V. vexillata	*angustifolia*	N.I. 522	0.15
V. vexillata	*macrosperma*	N.I. 339	0.15

dose 0.75%). Even at low concentrations (0.075–0.1%), PAPA markedly increased development times (MED) of both species, indicating ecologically significant sub-lethal effects of the chemical.

This study demonstrates that PAPA is approximately four and seven times as toxic [9] as the related structures L-DOPA and phenylalanine, respectively. The non-protein amino acid L-DOPA, which is accumulated in seed of several *Mucuna* spp. at concentrations between 5 and 10%, has been implicated as a chemical defence against bruchids and other seed predators [13], whilst the protein amino acid phenylalanine is a metabolic requirement for most insects, involved in the stabilization of cuticular proteins at ecdysis [14, 15], although it, too, can be toxic to bruchids if incorporated into the diet above a level of 5% [9]. The small number of wild *Vigna* species in *Plectotropis* and *Vigna* which accumulate PAPA appear to have evolved a chemical defence strategy based on the production of a taxonomically restricted toxic compound, which is effective against generalist seed predators even at relatively low concentrations in the seed. Chemicals which confer resistance to both Old World and New World pest bruchids are uncommon in seeds of wild and cultivated species from the *Vigna*/*Phaseolus* complex [10], i.e. seed of many Old World *Vigna* species are often more resistant to attack by New World than by Old World bruchids which may have co-evolved detoxification or avoidance mechanisms to counter chemicals to which they have been previously exposed over a long evolutionary timescale. This may explain why PAPA is less toxic to *C. maculatus* than to *Z. subfasciatus*, since both *C. maculatus* and the *Vigna* species accumulating PAPA are of Old World origin. *Callosobruchus maculatus* might therefore be expected to have a greater tolerance of a chemical which it may well have encountered during the long co-evolution of the bruchid with its legume hosts [11]. This is especially likely for more host-restricted (non-pest status) bruchids. From host range records [16] it appears that only one bruchid, *Specularius maindroni*, attacks *V. vexillata* seed, and it is likely that this specialist beetle has evolved a counter-adaptation to PAPA by developing a more

Table 3. Results of seed bioassays to measure total resistance to *C. maculatus* and *Z. subfasciatus*

		Resistance to *C. mac.*			Resistance to *Z. sub.*		
Vigna sp.	Accession	Ov.*	MED.†	Adults‡	Ov.*	MED.†	Adults‡
V. vexillata	TVNu 84	8	—	0	3	—	0
V. vexillata	TVNu 72	3	—	0	2	—	0
V. vexillata	CPI 17457	4	—	0	3	—	0
V. vexillata var. *vexillata*	NI 460	2	—	0	2	—	0
V. vexillata var. *angustifolia*	NI 522	2	—	0	2	—	0
V. vexillata var. *macrosperma*	NI 339	15	—	0	3	—	0
V. kirkii	NI 448	4	22.3	1.0	0	—	0
V. lobatifolia	NI 546	15	—	0	10	—	0
V. angivensis	NI 378	2	—	0	2	—	0

*Number of eggs laid per seed.

†Mean emergence day. —, MED not recorded due to 100% mortality during 1st–2nd instars.

‡Number of adults emerging per seed.

Table 4. Toxicity of PAPA to larvae of *Callosobruchus maculatus*

% PAPA in diet	MED ± s.e.*	Mean no. of adults emerging per capsule ± s.e.
0	27.5 ± 0.5	10.1 ± 0.3
0.075	33.8 ± 1.6	7.2 ± 0.2
0.25	34.2 ± 1.5	3.3 ± 0.1
0.50	35.7 ± 1.5	3.2 ± 0.1
0.75	—	0 ± 0

*MED, Mean emergence day ± standard error. —, Zero emergence; not calculable.

Table 5. Toxicity of PAPA to larvae of *Zabrotes subfasciatus*

% PAPA in diet	MED ± s.e.*	Mean no. of adults emerging per capsule ± s.e.
0	30.0 ± 0.5	4.0 ± 0.5
0.1	32.0 ± 1.2	2.4 ± 0.4
0.3	—	0 ± 0

*MED., Mean emergence day ± standard error. —, Zero emergence; not calculable.

specialized detoxification system than that used by generalist feeders. Specialist bruchids are known to attack seeds accumulating such insecticidal phytochemicals as rotenoids [17], alkaloids [9, 18] and non-protein amino acids [19], all of which are toxic to generalist beetles such as *C. maculatus* at naturally occurring concentrations. Many legume seeds do not rely on one type of chemical defence and may accumulate several chemicals of one class or compounds of several classes to increase their defence levels [9, 11, 20]. The high resistance of most *Plectotropis* species to *C. maculatus*, which cannot be explained by their PAPA content alone, may be due to the presence of other known antibruchid chemicals which have been detected in their seed, including trypsin inhibitors and phytohaemagglutins (lectins) [10]. Such multiple chemical defences may be additive or synergistic in action and protect seeds from predation by even the more host-restricted specialist bruchids [9, 17].

EXPERIMENTAL

Semi-quantitative measurement of PAPA in Vigna *seeds.* Finely ground seed (200 mg) was shaken with 1 ml 75% aq. MeOH for 24 hr, filtered and the filtrate (40 kl) subjected to ionophoresis on Whatman No. 1 paper (70 V/cm for 30 min) at pH 3.6 [21]. Papers were then dried and developed with ninhydrin (0.2% w/v in 95% aq. Me_2CO) or Ehrlich reagent (1% *p*-dimethylaminobenzaldehyde in a mixture of Me_2CO–HCl (9:1)). Spot colour intensities were then compared with a concn range of solns containing commercially purchased PAPA (Aldrich Co. Ltd., U.K.). Limits of detection for both reagents were 0.01% PAPA.

Quantitative estimation of PAPA in seed of V. vexillata. Finely ground seeds of each accession were extracted in 75% aq. EtOH (100 mg/ml). 0.3 ml of this extract was applied to 1 ml Amberlite CG-50 ion exchange resin (H^+ form) and washed with 4.0 ml H_2O. The column was eluted with 2.0 ml 2 M HOAc and the concn of PAPA determined in the HOAc eluate using the method of Seki *et al.* [22]. (Recovery of PAPA from the column was found to be 100%.) Detection limits of PAPA for this method were also 0.01%.

Bioassay of PAPA as a toxic resistance factor to bruchid larvae. In order to measure the toxicity of PAPA to larvae of the Old World pest bruchid *C. maculatus* and New World pest *Z. subfasciatus*, commercially obtained PAPA was added to artificial seeds containing ground cowpea flour [17] at a range of naturally occurring concns, up to a level causing 100% larval mortality.

Bioassay to measure total seed resistance to bruchids. Seed resistance to bruchids *C. maculatus* and *Z. subfasciatus* was quantified using lifetable studies to measure critical stages in the insect–host relationship. Oviposition, mean emergence day (MED) and the number of adults emerging per g of seed were recorded under controlled environmental conditions [10].

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