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Role of Endogenous Flavonoids in Resistance Mechanism of *Vigna* to Aphids

Vincenzo Lattanzio,^{*,†} Salvatore Arpaia,[‡] Angela Cardinali,[§] Donato Di Venere,[§] and Vito Linsalata[§]

Istituto di Orticoltura e Colture Industriali, CNR, Via S. Loja, Zona Industriale, 85050 Tito Scalo (PZ), Italy; Metapontum Agrobios, S.S., Ionica 106 Km 448.2, 75010 Metaponto (MT), Italy; and Istituto sull'Orticoltura Industriale, CNR, Via Amendola 165/A, 70126 Bari, Italy

Cultivated and wild species of the genus *Vigna* were screened for their flavonoid content. Flavonoid HPLC analyses clearly showed that cultivated lines of cowpea (*Vigna unguiculata* L. Walp.) are very similar from a qualitative point of view, always showing three flavonoid aglycons: quercetin, kaempferol, and isorhamnetin. In addition, a positive relationship between resistance/susceptibility characteristics against aphids and flavonoid glycoside content of cowpea lines was found. The resistant lines showed a flavonoid content higher than that of susceptible ones. In vitro bioassays proved that, among endogenous flavonoids, quercetin and isorhamnetin possess a good inhibitory aphid reproduction rate. Flavonoid HPLC analyses of wild *Vigna* species supported evidence for the existence of different flavonoid chemotypes in some species of section *Vigna*. There are kaempferol chemotypes, kaempferol being the main aglycon detected, quercetin chemotypes, containing quercetin glycosides only, and two isorhamnetin chemotypes. When the resistance characteristics to aphids in different chemotypes of the same species were tested, it became evident that quercetin or isorhamnetin chemotypes showed a higher level of resistance compared to kaempferol chemotypes in the same species, thus demonstrating a direct involvement of quercetin or isorhamnetin in the resistance mechanism. These results can provide useful information for further studies on gene expression of resistance factors.

Keywords: Plant phenolics; flavonoids; bioassay; aphid resistance; *Vigna* species

INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp.) is an important food legume in many countries in sub-Saharan Africa and Latin America. Its yield potential is very high (1.5–3.0 t/ha), but the actual yields in much of the developing world are far less, averaging 0.2–0.4 t/ha. The major constraints to cowpea production are insect pests, plant diseases, plant parasitic weeds, drought, and heat (Murdock, 1992; Singh et al., 1992; Thottappilly et al., 1992).

There are two *Aphis* spp. (Homoptera: Aphididae) reported as pests of cowpeas: *A. craccivora* Koch, which is the main aphid infesting cowpeas throughout Africa and Asia, and *A. fabae* (Scopoli), which has been reported as a minor pest in Africa and whose biology appears to be similar to that of *A. craccivora*. Cowpea aphids primarily infest seedlings, but large populations also infest flowers and green pods of older plants (Annan et al., 1996; Singh and Jackai, 1985). The ecological relationship between plants and insects is complex one with physical as well as chemical interactions. This relationship is also affected by plant factors, insect factors, and some insect–plant factors, including hypersensitive reaction and plant resistance to insect-

borne disease. Various environmental conditions can modify the expression of these factors by acting primarily on the insect, the plant, or the insect–plant relationships. Each of the plant or insect mechanisms indicated may be the result of one or more genetic factors (Painter, 1941).

Plant volatiles and visual and thigmotactic cues may be involved in an insect's recognition of, and migration to, a host plant. After locomotion is arrested, probing occurs. The net result of individual feeding stimuli and deterrents determines whether the insect will remain and feed. Whether a plant is accepted or rejected as food by insects depends largely on its chemical composition in addition, of course, to physical factors such as toughness, thickness, and hairiness. In addition, chemical inhibitors play an important role in the inhibition of oviposition on the host-plant and, in turn, in insect larval growth and survival of progeny (Chapman, 1974; Dethier, 1970; Stotz et al., 1999). Studies on the role of inhibitors in host-plant selection indicate that many different chemicals may be expected to have an inhibitory effect on feeding by different insects. Among plant constituents, phenolics are known to be involved in disease and insect resistance of crop plants (Corcuera, 1993; Elliger et al., 1980; Todd et al., 1971). Plant flavonoids affect the behavior, development, and growth of a number of insects (Hedin and Waage, 1986). Some cotton flavonoids are feeding stimulants for the boll weevil, *Anthonomus grandis* (Hedin et al., 1988), or oviposition stimulants of a *Citrus*-feeding swallowtail butterfly, *Papilio xuthus* L. (Nishida et al., 1987), or,

* Author to whom correspondence should be addressed (telephone +0390971427249; fax +390971427222; e-mail lattanzio@ioci.pz.cnr.it).

[†] Istituto di Orticoltura e Colture Industriali.

[‡] Metapontum Agrobios.

[§] Istituto sull'Orticoltura Industriale.

finally, antibiotic substances efficient against phytophagous insects (Chan et al., 1978; Chan and Waiss, 1978; Elliger et al., 1980; Hanny, 1980; Hedin et al., 1983; Joerdens-Roettger, 1979; Peng and Miles, 1988; Ridsdill-Smith et al., 1995; Todd et al., 1971).

Previously, cowpea lines with high levels of resistance to cowpea aphid have been identified. Several cultivated lines of cowpea, including TVu 36, TVu 310, TVu 408P₂, TVu 801, and TVu 3000, are resistant to aphid infestation (Singh and Jackai, 1985; Singh et al., 1992). To identify higher levels of resistance, several wild *Vigna* species have been screened for resistance to aphids: *V. reticulata*, *V. racemosa*, and *V. angustifolia* were found to be aphid resistant (Singh et al., 1990). Wild species are known to be a source of useful genes, mainly for resistance or tolerance to diseases, pests, and abiotic factors not found in cultivated species. This higher level of resistance that characterizes wild species or ecotypes derives from their ability to withstand extreme environmental conditions. Through hybridization between cultivars that differ genetically and subsequent selection from the hybrid populations, plant breeders have been very effective in increasing the production of cultivated food crops. Nevertheless, to date, the introduction of resistant genes from wild species to cowpea is constrained by barrier to crossing between species. A better understanding of the degree of genetic affinity between cowpea and wild *Vigna* species would enhance the use of the *Vigna* gene pool. In addition, there is the route of genetic engineering: if genes coding for resistance to one or more of the cowpea insect pests could be identified, cloned, and introduced in cowpea, a susceptible cowpea plant could be transformed into a resistant plant (Thottappilly et al., 1992).

Previous papers have described the characterization of the phenolic fraction in leaf extracts of *Vigna unguiculata* (L.) Walp. lines and some wild species of *Vigna* (Lattanzio et al., 1990, 1996). The phenolic aglycons identified were vanillic acid, *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid, kaempferol, quercetin, isorhamnetin, and apigenin. The ubiquitous cinnamic acid derivatives, *p*-coumaric acid being the most abundant among them, are of little taxonomic value. The present paper deals with the chemical nature of the inhibitory substances in leaves of *Vigna* species, their role in the resistance mechanism against aphids, and, finally, the potential utilization of wild species of *Vigna* as sources of useful genes for resistance or tolerance to insect pests.

MATERIALS AND METHODS

Plant Material. Cultivated and wild species belonging to the genus *Vigna*, subgenera *Vigna* (sections *Catiant* and *Vigna*), *Plectotropis* (section *Plectotropis*), and *Ceratotropis*, were analyzed. All seed samples were supplied by the International Institute of Tropical Agriculture (IITA) located at Ibadan, Nigeria. All species were grown from seed in a growth chamber at 25 °C (RH = 70%) with a 16 h/8 h light–dark photoperiod up until the six leaf was initiated. The youngest leaves from the tip were collected from single plants and analyzed for phenolic compounds.

Analysis of Phenolic Compounds. For qualitative determination of the flavonoids, the plant material was refluxed with hot methanol/ethanol/water (MeOH/EtOH/H₂O) (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 aglycon analysis, the aqueous ex-

tracts were first hydrolyzed under nitrogen with 0.3 M HCl and then extracted with diethyl ether (Et₂O). Finally, the Et₂O extracts were concentrated under vacuum and redissolved in MeOH. The latter were analyzed by HPLC.

HPLC analyses were performed with a Perkin-Elmer series 4 liquid chromatograph, which was equipped with a fluorometric detector PE-LS3 and a computer-aided Hewlett-Packard spectrophotometric photodiode array detector 1040-A, 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 an HP-K3 software post-run analysis coupled with PE-Chromatographics 2 software. The pilot signal to the spectrophotometric detector was set at 325 nm and the fluorometric detector at 330 nm (excitation) and 390 nm (emission). An analytical Waters (Milford, MA) column (300 mm × 4 mm i.d.) packed with μ Bondapak C18 (10 μ m) was used throughout this work. The solvent system consisted of (A) MeOH and (B) acetic acid/water (5:95, v/v).

Bioassays. All phenolic aglycons identified in *Vigna* species were tested on *Aphis fabae* (Scopoli) for their in vitro inhibitory activity. The aphid colony was originated from individuals collected in the field on *Beta vulgaris* and maintained on *Vicia faba minor* in a growth chamber ($T = 25$ °C; RH = 70% and a 16 h/8 h light–dark photoperiod). Leaves of faba bean were set with the cut petiole in water (reference) or 0.1 mM 10% methanolic solution of phenolic compounds. After 12 h of imbibition, three adult aphids were placed on each leaf by using a small (no. 1) camel hair paintbrush, and then the number of larvae was counted daily with the use of a magnifying lens.

In vivo bioassays were carried out by utilizing five 1-week-old *Vigna* plants infested with seven adults of *A. fabae*. Plants were placed in a growth chamber at 25 °C with a 16 h/8 h light–dark photoperiod. The number of nymphs was counted daily for 3 days. The effect of host plant on nymph deposition was scored using a repeated-measurements ANOVA, with unbalanced design. Separation among treatments was executed with Ryan's *Q* test ($p = 0.01$).

RESULTS AND DISCUSSION

In the present investigation cultivated and wild species of *Vigna* were screened for their phenolic content. Flavonoid HPLC analyses clearly showed that cultivated lines of *V. unguiculata* L. Walp. are very similar from a qualitative point of view, containing always three flavonoid aglycons: quercetin, kaempferol, and isorhamnetin. The flavonoid glycoside patterns of the analyzed lines were similar and showed 10 different glycosides, among which were found *p*-coumaroylglycosides of kaempferol and quercetin. In addition, a positive relationship between resistance/susceptibility characteristics against aphids and flavonoid glycoside content of cowpea lines was found. The resistant lines showed a flavonoid content higher than that of susceptible ones. This relationship was further confirmed when the flavonoid aglycon contents of two near-isogenic lines of *V. unguiculata* were considered: the level of flavonoids in IT 84-E-1-108 (resistant) was twice as high as in IT 82-E-60 (susceptible) (Figure 1).

On the basis of these observations, it was argued that leaf flavonoids could be involved in the resistance, acting as inhibitors of aphid reproduction. Therefore, there was a need for a bioassay to identify the antifeeding compound(s) involved in resistance. Because some *Vigna* endogenous phenolics are poorly soluble in water, it was necessary initially to find how to solubilize water-insoluble compounds without adversely affecting aphid feeding. Figure 2 shows the effect on aphid feeding behavior of methanol tested at five concentrations (0, 1, 2, 5, and 10% in water) using the single cut leaf

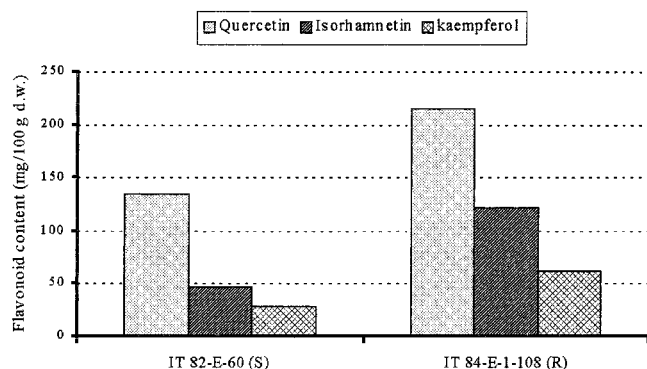


Figure 1. Flavonoid content in NILs of *V. unguiculata* (L.) Walp. (S = susceptible; R = resistant).

bioassay. On the basis of these experiments, a 10% aqueous methanol solution was selected as solubilizing agent for phenolic compounds in bioassays because this solution was superior as a solvent but had no adverse effect on aphid feeding. Figure 3 shows the inhibitory effect of *Vigna* endogenous phenolics, relative to the control, upon nymph deposition by *A. fabae*. The most inhibitory compound was found to be vanillic acid, but

its content in *Vigna* leaves is very low. As to the inhibitory activity of flavonoid aglycons, quercetin possesses the highest activity, whereas kaempferol shows little activity on reproduction rate. Unfortunately, no data are available to date on the effect of unidentified *p*-coumaroylglycosides of quercetin on aphids. *p*-Coumaric acid is a good in vitro inhibitor of aphid reproduction, but its plant tissue content is not positively correlated with resistance in cultivated *Vigna* lines. In conclusion, the results of this investigation indicate that there exists in cowpea aphid a physiological/biochemical system vulnerable to selected *Vigna* leaf flavonoids. The mechanisms by which insects react to, or interact with, plant flavonoids are multiple: antibiotic flavonoids can also be shown to induce, in a different situation, a nonpreference mechanism. In structure–activity research it has been shown that O-dihydroxylation in either the A or B ring increases the inhibitory activity of flavonoids and that the position of glycosidation (3-*O*- or 7-*O*-glycosides) and the nature of the sugar also affect the effectiveness of flavonoids as antimicrobial agents (Chen et al., 1999; Elliger et al., 1980; Jones and Klocke, 1987; Laks and Pruner, 1989; Morimoto et al., 1999; Todd et al., 1971; Wang et al., 1989).

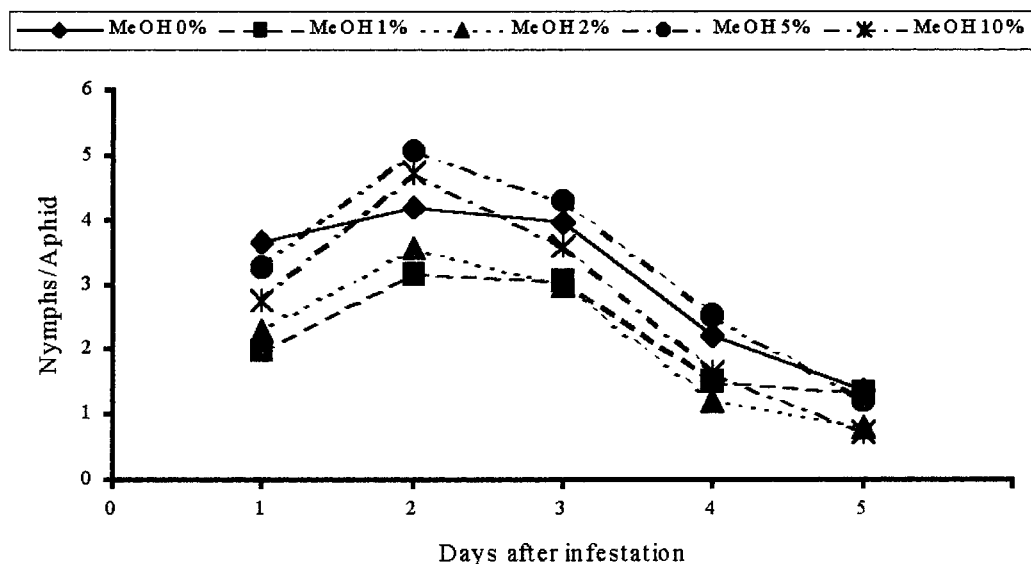


Figure 2. Effect of methanol concentration on daily nymph deposition by *A. fabae*.

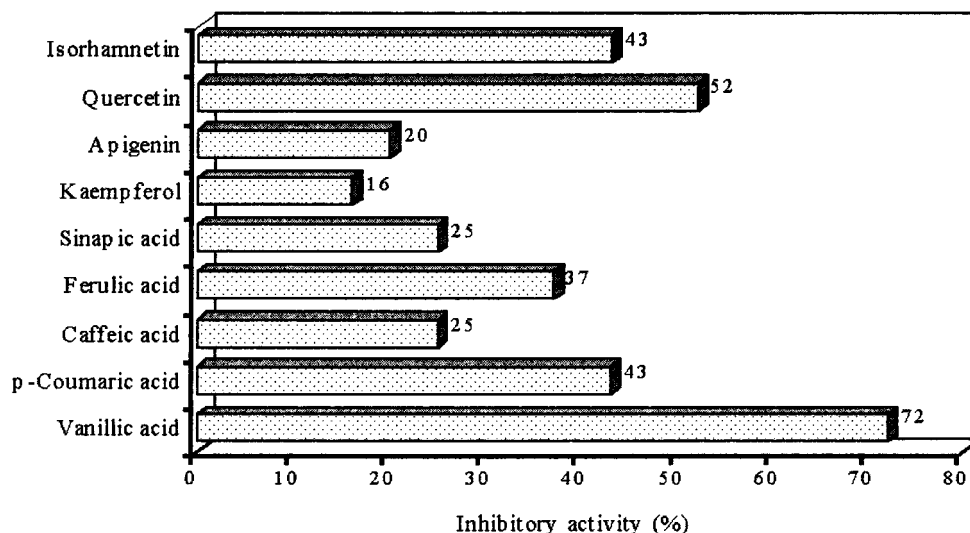


Figure 3. Inhibitory activity of phenolics (0.1 mM in 10% MeOH) on *A. fabae* progeny deposition.

Table 1. Daily Production of *A. fabae* Nymphs on *Vigna* Accessions

accession	larval daily deposition	Ryan's <i>Q</i> ($p = 0.01$)
kaempferol chemotype		
<i>V. luteola</i> (Jacq.) Bentham Tvnu 172	7.73	A
<i>V. marina</i> (Burm.) Merrill var. <i>marina</i> Tvnu 717	7.25	A
quercetin chemotype		
<i>V. luteola</i> (Jacq.) Bentham Tvnu 475	1.50	B
isorhamnetin chemotype		
<i>V. marina</i> (Burm.) Merrill var. <i>oblonga</i> Tvnu 174	0.67	B

The quantity of the inhibitory flavonoids may be of great importance in the resistance of crop plants to insects; however, the qualitative difference should limit the range of host plants for insects (Chan and Waiss, 1978). In addition, there seems to be no reason to believe that resistance relationships found in domesticated plants do not also occur among wild ones and that the characteristics such as flavonoid content, which taxonomists use to separate species, can hardly have enough adaptive value for survival through natural selection.

In this research work flavonoid HPLC analyses of wild *Vigna* species supported evidence for the existence of different flavonoid chemotypes in some species belonging to section *Vigna*. There are kaempferol chemotypes, in which kaempferol was the only or the main aglycon detected, quercetin chemotypes, containing only quercetin glycosides, and two isorhamnetin chemotypes. From an ecological point of view the most interesting chemotypes are some accessions, belonging to the same species, which permit the study of, *ceteris paribus*, the role of endogenous flavonoids in plant resistance to aphids. Among *V. marina* accessions two chemotypes were found: *V. marina* var. *oblonga* TVnu 1174 (isorhamnetin chemotype) contained two isorhamnetin glycosides and traces of two kaempferol glycosides, whereas *V. marina* var. *marina* TVnu 717 (kaempferol chemotype) contained only kaempferol glycosides. *V. luteola* accessions also showed two different chemotypes: the accession TVnu 475 contained only quercetin glycosides, whereas the other two accessions, the kaempferol chemotypes TVnu 172 and TVnu 905, were similar from a qualitative point of view with some quantitative differences in the flavonoid glycoside pattern—both contained robinin (kaempferol-3-robinoside-7-rhamnoside).

When the resistance characteristics to aphids in different chemotypes of the same species were tested (Table 1), it became evident that quercetin or isorhamnetin chemotypes showed a higher level of resistance compared to the kaempferol chemotypes of the same species, thus demonstrating a direct involvement of quercetin or isorhamnetin in the resistance mechanism. These results can provide information useful in the development of studies on gene expression of the resistance factors.

In conclusion, the fact that insects may be prevented from feeding by enhancing certain chemicals in plant composition is of potential value in crop protection: a plant that has repellent or deterrent properties will inhibit continuous feeding by an insect so that it tends to become active and move away (Chapman, 1974; Stotz et al., 1999). The existence of different chemotypes expressing a presence/absence character, with regard to inhibitory flavonoid (quercetin or isorhamnetin), in the same species permits development of resistant varieties, thus eliminating or reducing synthetic insecticides.

This could be accomplished by means of two possible approaches: the biotechnological approach and selective breeding programs overcoming the sexual barrier to hybridization between wild *Vigna* species and cultivated ones. A backcross program could be carried out with the aim of obtaining nearly isogenic lines (NILs). Starting from these genotypes the "differential display" technique could be used (Hannappel et al., 1995), which permits the identification in different chemotypes (resistant and susceptible to aphid attack) of differentially expressed mRNAs. It should be therefore possible to isolate expressed genes from these regions of the genome that differ among NILs. In addition, the somatic hybridization technique, via protoplast manipulation, could allow a breeding approach without gene isolation methodology.

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