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Seed coat tannins and bruchid resistance in stored cowpea seeds

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Abstract: Seeds of wild species and varieties of *Vigna* were screened for their tannins and α-amylase inhibitor contents as defensive compounds against cowpea weevil. Seed coats contained condensed tannins that were positively correlated to their colour but not to their resistance against the insect. The α-amylase inhibitors were present in different amount in cotyledons of all species analysed. Amongst the cultivated lines assayed, *Vigna unguiculata* TVu 2027, an accession identified as moderately resistant, was found to contain the higher amount of α-amylase inhibitor. When wild species were considered, *V luteola* and *V vexillata* (two resistant species) showed the highest content of α-amylase inhibitors. In addition, two cultivated accessions (Vita 7 and IT 84E-1-108) of cowpea seeds, both classified as susceptible accessions, showing a different degree of bruchid damage in storage, were also analysed. No α-amylase inhibitory activity was found in cotyledons of undamaged Vita 7 seeds, while the seed coat tannin content was found to be 13 times higher in undamaged Vita 7 seeds than in IT 84E-1-108 infested seeds. These latter results support the hypothesis that seed coat tannins must also be considered in biochemical defence mechanisms, which can deter, poison or starve bruchid larvae that feed on cowpea seeds.

Keywords: *Vigna*; condensed tannins; α-amylase inhibitors; *Callosobruchus maculatus*; host–plant resistance mechanism

INTRODUCTION

Cowpea [*Vigna unguiculata* (L) Walpers] is a tropical legume crop of African origin and an essential component of cropping systems in the drier regions and marginal areas of the tropics and subtropics. The culture of cowpea is particularly important in West Africa where over 9.3×10^6 ha are covered, leading to 2.9×10^6 tonnes of annual production.[1]

Cowpea suffers heavily from insects, both in the field as well as when the grain is stored after harvest. The most important insect pests in cowpea production systems are probably aphids (*Aphis craccivora*), pod borers (*Maruca vitrata*), thrips (*Megalurothrips sjostedti*) and the pod-sucking bug *Clavigralla tomentosicollis*.[2,3] The cowpea seed beetle, sometimes also known as the cowpea weevil, although not a member of the family Curculionidae, *Callosobruchus maculatus* (F), is a major pest of stored cowpeas, but actually infests the green pods while still in the field. The adult beetles lay eggs on drying cowpea pods in the field and/or seeds in storage. Larvae hatching from eggs use their mouthparts to penetrate the pod wall or the seed testa. Larval feeding in the cotyledons causes significant losses in seed weight, germination viability and seed marketability.[4–8]

There are many varieties of cowpeas and they show a wide range of chemical and physical characteristics such as seed colour, texture, size, hardness and chemical constituents.[9] All these characteristics, as well as the existence of intraspecific variation among populations of *C maculatus*, have been considered in different research about resistance of cowpea varieties and/or species to bruchid attack. It has been suggested to use such physical factors to complement the biochemical factors found in bruchid-resistant cowpea varieties.[4,10–12]

By screening a world germplasm collection, a moderate level of resistance to cowpea bruchid has been identified in TVu 2027 accession and the mechanism of resistance was found to be antibiosis, resulting in larval mortality.[8] Thereafter, in order to identify higher levels of resistance, many wild *Vigna* species have been screened for bruchid resistance, thus identifying several resistant species (*V luteola*, *V vexillata*, *V oblongifolia*, *V racemosa* and *V reticulata*).[12] The resistance to bruchids in TVu 2027 was

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investigated by Gatehouse *et al*,[13] who concluded that resistance was derived from an elevated level of trypsin inhibitor within the cowpea seeds. However, some researchers suggest that the trypsin inhibitor alone does not account for bruchid resistance in cowpea, thus indicating a need for further investigations.

Reports on the effect of seed coat on oviposition and survival of *C maculatus* are conflicting. For example, Nwanze and Horber[14] suggested that causes of resistance in cowpea to *C maculatus* might be categorized as non-preference during oviposition and antibiosis during larval development. Antibiosis may not only be explained as a biochemical phenomenon, but it also involves physical components, namely the surface texture and structure of the seed coat, which affect larval penetration. Cowpea weevil prefers smooth-coated seeds to wrinkled seeds for oviposition, and more first instar larvae successfully penetrate the seed coat in smooth than in rough seeds. In contrast, Edde and Amatobi,[11] in their experiments on 22 cowpea varieties (five resistant, four moderately resistant, and 13 susceptible varieties), with and without seed coat, observed that seed coat has no value in protecting cowpea seed against attack by *C maculatus*. However, even if it has been suggested that the growth and development of cowpea weevil depend on the nutritional value of the seeds, not enough data about the level of antinutritional factors in seed coat are available. In this connection Sales *et al*[15] suggested that the high levels of vicilins, also known as 7S storage globulins, expressed in the seed coat of cowpea accession TVu 2027 are enough to deter development of first instar larvae of *C maculatus*.

Birch *et al*[16] have focused on the significance of *p*-aminophenylalanine (PAPA) in *Vigna* as a seed defence against bruchids, but Bressan[17] demonstrated that *V vexillata* accessions that differed in resistance to bruchids did not have significantly different levels of PAPA in the mature seeds. Also lectins, a group of proteins possessing at least one non-catalytic domain which binds reversibly to a specific mono- or oligosaccharide, have been considered as defensive compounds against cowpea weevil, even if the toxic effects of active lectins in some cases could be due to an α-amylase inhibitor contaminant in the preparation used.[18–20] Plant α-amylase inhibitors are particularly abundant in cereals and leguminosae. Some wheat α-amylase inhibitors inhibit insect α-amylases strongly but do not inhibit mammalian α-amylases, suggesting that they could be used as tools of engineered resistance of crop plants against pests. Bean α-amylase inhibitors, when added in low concentrations (1%) to artificial diet, proved to be toxic to the larvae of cowpea weevil and adzuki bean weevil.[6,21–26]

Generally, 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. Therefore, the high resistance of some cultivated or wild *Vigna* species to *C. maculatus* may be due to the presence of multiple chemical factors with additive or synergistic action to protect seeds from predation. This paper deals with the evaluation and characterization of some antinutritional factors (seed coat tannins and cotyledon α-amylase inhibitors) in cowpea seeds and related wild species. Tannins, hydrolysable tannins and condensed proanthocyanidins are large polyphenolics whose molecular weights range from 500 to 4000 Da and whose many hydroxyl groups interact with proteins, denaturing and precipitating them from solution.[27] Tannins may affect the growth of insects in three main ways: they have an astringent taste which affects palatability and decreases feed consumption, they form complexes with proteins of reduced digestibility and they act as enzyme inactivators.[28] Seed tissues contain tannins located mainly in a layer between the outer integument and the aleurone layer, while α-amylase inhibitors are located in cotyledons. The major purpose of this study is the improvement of knowledge on the biochemical basis of defensive strategies of cowpea against bruchids, thus allowing plant breeders to increase the insect resistance of cultivated varieties.

MATERIALS AND METHODS

Seeds of *Vigna* species and accessions (Table 1) were obtained from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. On the basis of tests for insect resistance conducted at IITA, seeds were classified as resistant or susceptible to *C maculatus*.

Tannins extraction and assay

Tannin analysis was carried out on 10 g of dry seed coat from different cultivated *Vigna* lines according to Vande Casteele *et al*.[29] The extraction was performed with boiling methanol–ethanol–water (4:4:2, v/v/v) under nitrogen; the solvent was evaporated under vacuum and the residue dissolved in water (crude extract). Tannins were separated from non-tannins with 1% gelatin in 10% NaCl solution and the pellet, containing tannins, was solubilized in 50% pH 3 acetone at 37 °C. Gelatin was then removed, increasing the acetone concentration up to 90%. The acetone was evaporated under vacuum, total tannins were dissolved in water and the hydrolysable fraction separated from the condensed one using formaldehyde at 1.6 mg ml^{-1} final concentration. Tannins were assayed by Folin–Ciocalteau spectrophotometric method and results were expressed as catechin equivalents. The presence of condensed tannins was also proved by the red colour formation after heating the crude extracts with *n*-butanol–HCl–Fe(III).

α-Amylase inhibitors extraction

Ten grams of finely ground de-hulled seeds were extracted by being stirred overnight at 4 °C with 0.15 M NaCl (1:5, w/v), followed by centrifugation at 12 100 × *g* for 60 min. The supernatant was buffered

Table 1. List of *Vigna* species and/or accessions analysed for tannin and α-amylase inhibitor content and their origin

Species and/or accessions[a]	Origin
Vigna unguiculata (L) Walpers ssp *unguiculata* cg *unguiculata* Westphal (cultivated):	
Ife Brown (S)	Nigeria
TVu 9062 (nd)	Nigeria
TVu 1 (nd)	Nigeria
TVu 36 (S)	Nigeria
TVu 801 (S)	Nigeria
Vita 7 (S)	Nigeria
TVx 3236 (S)	Nigeria
TVu 3000 (S)	Nigeria
IT 83S-728-5 (S)	Nigeria
IT 83S-742-13 (S)	Nigeria
IT 84E-1-108 (S)	Nigeria
IT 82E-60 (S)	Nigeria
IT 82D-716 (R)	Nigeria
IT 84S-2246-4 (R)	Nigeria
IT 84S-2231-15 (R)	Nigeria
IT 84S-275-9B (R)	Nigeria
IT 81D-1020 (R)	Nigeria
IT 81D 1137 (R)	Nigeria
IT 81D-994 (R)	Nigeria
TVu 2027 (R)	Nigeria
Vigna unguiculata (L) Walpers ssp *dekindtiana* var *dekindtiana* TVnu 965 (S)	Nigeria
Vigna unguiculata (L) Walpers ssp *dekindtiana* var *dekindtiana* TVnu 413 (S)	Nigeria
Vigna ambacensis Baker var *ambacensis* TVnu 166 (S)	Central African Republic
Vigna ambacensis Baker var *ambacensis* TVnu 156 (S)	Central African Republic
Vigna oblongifolia A Richard var *oblongifolia* TVnu 37 (R/S)	Costa Rica
Vigna racemosa Hulch and Dalziel TVnu 181 (R)	Nigeria
Vigna luteola (Jacq) Bentham TVnu 475 (R)	Kenya
Vigna luteola (Jacq) Bentham TVnu 172 (R)	Brazil
Vigna luteola (Jacq) Bentham TVnu 905 (R)	Botswana
Vigna vexillata A Richard var *vexillata* TVnu 74 (R)	Rwanda
Vigna vexillata A Richard var *macrosperma* TVnu 64 (R)	Australia
Vigna vexillata A Richard var *vexillata* TVnu 72 (R)	Costa Rica

[a] Classification of susceptibility toward *C maculatus*, as determined by IITA. R, resistant; S, susceptible; nd, not determined.

by adding 0.2 M Na-succinate, 0.1 M $CaCl_2$ (pH 3.8) (110 µl ml^{-1}) and was heated in a water bath at 70 °C for 15 min. The protein precipitate was removed by centrifugation (12 100 × *g* for 60 min) and the clear supernatant was brought to pH 5.6 with NaOH. Ethanol, until 19% final concentration, was added to this solution and the mixture was stirred for 3.5 h at 4 °C and then centrifuged.[30,31] The supernatant (ethanol fraction) was used for further purification.

Preparation of insect α-amylase

Midguts dissected from the last instar larvae of *C maculatus* were homogenized in 0.03 M sodium barbital, 0.2 M sodium acetate, 0.44 M NaCl, 1 mM $CaCl_2$ buffer, pH 5.4 (50 µl midgut^{-1}) and centrifuged at 12 100 × *g* for 20 min. The supernatant was used as a larval α-amylase preparation. The α-amylase was diluted with the buffer solution (1:20 v/v) and used immediately.

α-Amylase inhibitor assay

The inhibitory activity assay was performed by adding different volumes of inhibitor extract to 50 µl of insect α-amylase preparation in a total volume of 1.2 ml of barbital buffer solution, pH 5.4. The mixture was incubated at 20 °C for 10 min before the addition of 0.2 ml of substrate solution (0.1% potato starch solution in water). After incubation at 20 °C for 10 min, the reaction was stopped with 0.2 ml 3 M HCl. The undigested starch was determined by adding 0.4 ml of an I_2–KI solution (1.2 and 1.8 mM, respectively) and by measuring the change in absorbance at 620 nm. Controls without inhibitors were included to determine amylase activity of each preparation (expressed as amylase units, ie the amount of enzyme that gave 50% hydrolysis of the added starch).[26] The α-amylase inhibitory activity (percentage of control) was expressed as percentage of α-amylase activity values in the absence of pre-incubation with the seed extract. The Bradford method was used for protein determination of α-amylase inhibitor extracts using bovine serum albumin as a standard.[32]

Electrophoresis

SDS–PAGE was performed by two methods. The first, according to Schagger and Von Jagow,[33] used 4% (w/v) polyacrylamide in the stacking gel, 10% in

the spacer gel and 14% in the separating gel. Running conditions were: anode buffer—0.2 M Tris, pH 8.9; cathode buffer: 0.1 M Tris, 0.1 M tricine, 0.1% SDS, pH 8.25. The second method, according to Payne,[34] used 3% (w/v) polyacrylamide in the stacking gel and 19% in the running gel. The running buffer was 0.25 M Tris, 1.88 M glycine, 1% SDS, pH 8.3. Gels were stained overnight at room temperature with 0.25% Coomassie blue R-250 in 10% acetic acid and were destained with 10% acetic acid solution.

α-Amylase inhibitor purification by affinity chromatography

Purification of α-amylase inhibitor was done by affinity chromatography on Procion Red Sepharose CL-6B;[35] the gel was prepared according to the manufacturer's instruction (Amersham). The bead was equilibrated with 0.05 M Tris–HCl, 0.5 M NaCl pH 7 buffer and then the inhibitor extracts were loaded on a chromatographic column. The non-binding fractions were eluted with the starting buffer instead of the specifically bound proteins, with 0.05 M Tris–HCl, 3 M NaCl pH 7. The pooled fractions showing α-amylase inhibitor activity were concentrated to 2 ml by ultrafiltration on Amicon YM1 membrane and used for further studies.

RESULTS AND DISCUSSION

Several cowpea accessions with different resistance characteristics have been screened for their seed coat tannin content (Fig. 1). All the analysed accessions contained only condensed tannins (proanthocyanidins), whose content was positively correlated with the seed coat colour, while no relationship between the tannin level in seed coats and resistance characteristics was found. Similar results were also found by Baker *et al*.[36] It is interesting to emphasize that cowpea line TVu 2027, an accession classified by IITA as a moderately bruchid-resistant line, showed a very low concentration of tannins while VITA 7, a susceptible line, contained a very high concentration of tannins.

These results are in good agreement with the suggestions of some authors,[11,37] indicating that the chemical characteristics of seed coat did not have an influence on oviposition and larval development of cowpea weevil but the resistance factors are carried in the cotyledon and embryo of the seed. On the other hand, the same results might suggest some reflections. Firstly, the classification made by IITA, concerning resistance characteristics of samples analysed, cannot be considered a dogma: many problems are encountered with analysis of the results because of the variability of the insects' development and the natural variability in sample susceptibility that greatly affect the reliability of conventional and innovative techniques for screening cowpea varieties for resistance to the bean weevil. In addition, sometimes a complete disagreement was observed amongst results coming from the different bioassay techniques used to differentiate resistance characteristics of seeds.[38] This reflection might also explain, for example, why Baker *et al*[36] by screening 15 lines of cowpeas, *C maculatus*-susceptible or -resistant, contrary to claims that elevated trypsin inhibitor levels may correlate with *C maculatus* resistance,[13] observed that trypsin inhibitor activities in the seeds did not differ significantly between resistant and susceptible lines. Secondly, the opinion of the authors is that seed coat proanthocyanidins cannot be considered insignificant as far as larval survival and penetration is

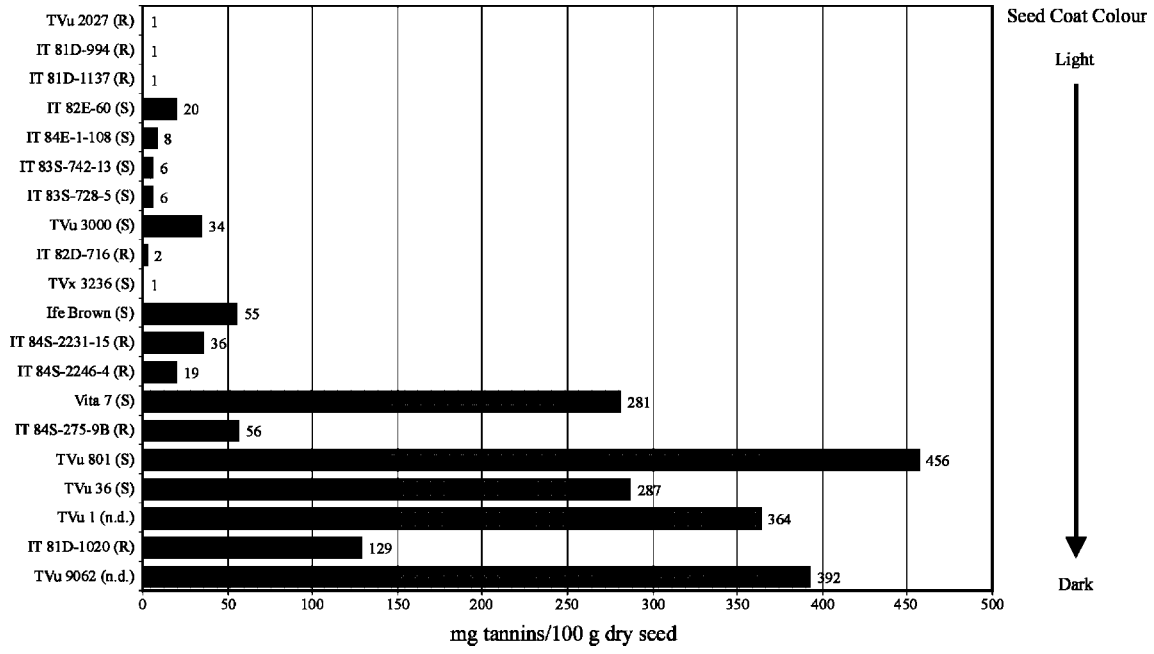

Figure 1. Proanthocyanidin contents of cultivated accessions of *Vigna unguiculata* (L) Walpers (data are expressed as mg 100 g^{-1} dry seed, applying catechin calibration).

concerned. Even if the penetration of cowpea testa by bruchid larvae is prevalently mechanical,[39] the properties of seed coat proanthocyanidins in a no-choice situation may influence food breakdown, thus affecting the ability of larvae to infest seeds.[40]

However, before developing this latter aspect, the second seed defence compound must be considered. As stated above, in fact, the resistance characteristics of plant tissues are the result of a set of defence mechanisms acquired by plants during evolution. Besides antibiotics such as tannins, seed defence includes some storage proteins, such as enzyme inhibitors, that act on key insect gut digestive hydrolases. α-Amylase inhibitors of legume seeds, located in cotyledons, are detrimental to the development of cowpea weevil, *C maculatus*, as this insect is highly dependent on starch as an energy source.[41,42] Therefore, cultivated lines of cowpea and related wild species of *Vigna* were analysed for their α-amylase inhibitor content. A partially purified preparation of α-amylase inhibitor was obtained from seed powders by means of 0.15 M NaCl extraction, heat treatment and ethanol precipitation. After the first three steps of purification the protein recovered was the 3–5% of the total protein detected in the dry seed. No inhibitory activity was detected in the discharged protein. Figure 2 shows the α-amylase inhibitory activity in the ethanol fraction of wild and cultivated species or accessions of *Vigna* of different origins. Amongst the cultivated cowpeas, the inhibitory activity in TVu 2027, an accession showing a moderate level of resistance, was found to be seven times higher than in VITA 7, a susceptible line. Amongst the analysed wild species, *V luteola* and *V vexillata* are known to be resistant against *C maculatus* while *V dekindtiana* and *V ambacensis* are susceptible ones.[8] Figure 2(a) shows that, generally, the α-amylase inhibitory activity in *V luteola* and *V vexillata* is very much higher than in susceptible lines, even if some resistant accessions showed an inhibitory activity lower than the susceptible accessions. When the amount of α-amylase inhibitor that gives 50% inhibition of insect α-amylase is considered, *V luteola* TVnu 905 and *V vexillata* TVnu 74 need 5.6 and 11.2 µg of protein, respectively. On the other hand, *V ambacensis* TVnu 156 and *V dekindtiana* TVnu 965 need 40.2 and 88.0 µg of partially purified α-amylase inhibitor. However, the figure also shows that, amongst the different accessions of the same species, a great variability in the inhibitory activity can be detected.

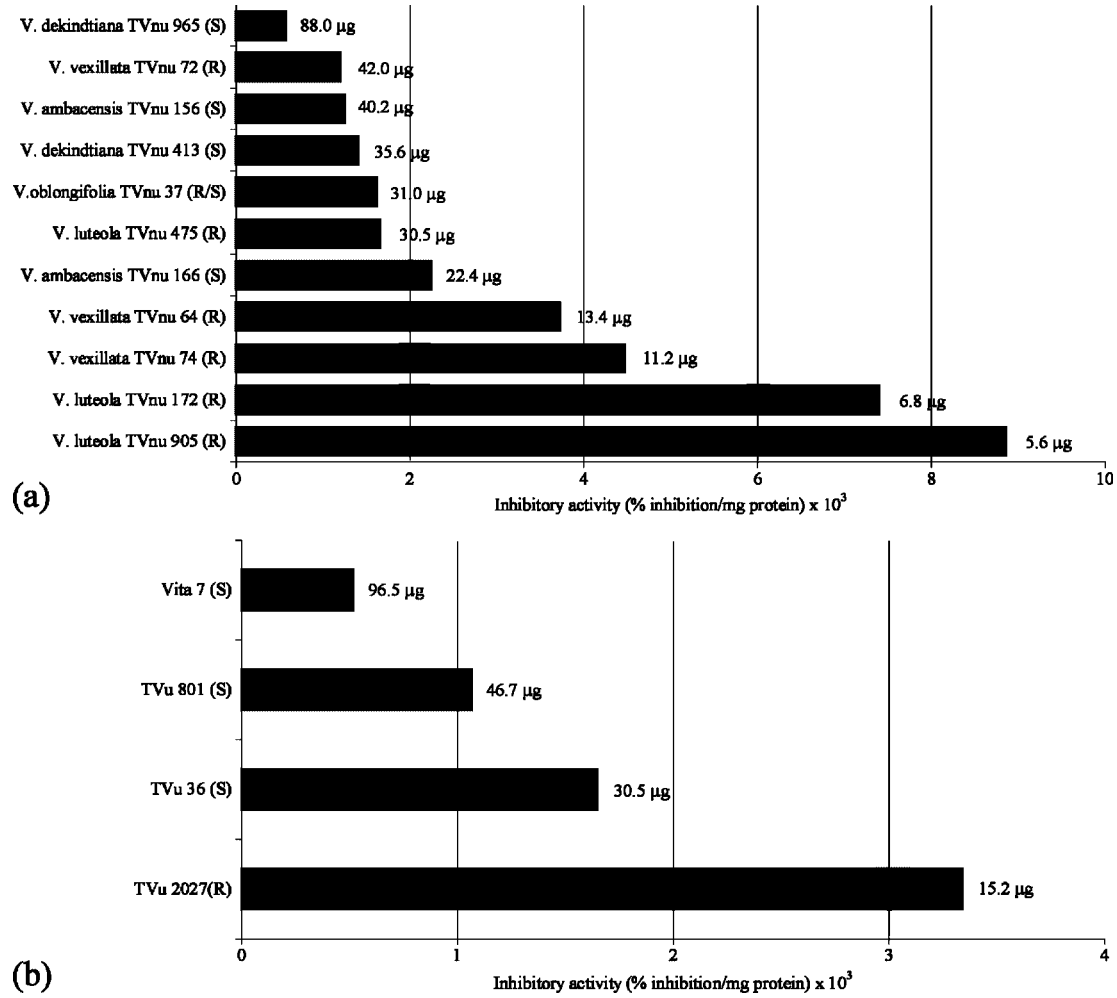

Figure 2. α-Amylase inhibitory activity in wild (a) and cultivated (b) species and accessions of *Vigna* (values in figure represent µg of protein that gives 50% inhibition of insect α-amylase).

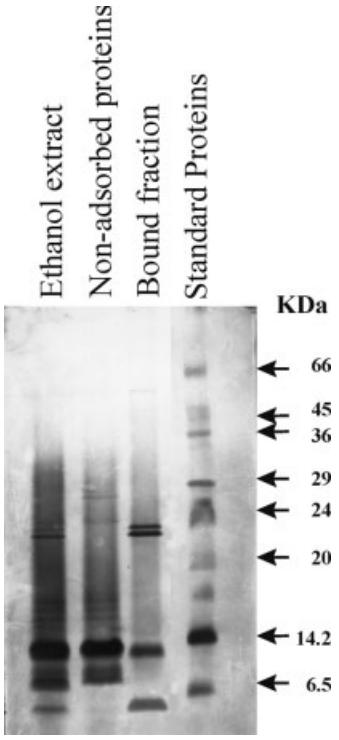

Figure 3. SDS–PAGE of ethanol extract and eluate from Red-Sepharose affinity chromatography.

Further purification of the α-amylase inhibitors was carried out on the ethanol fraction of *V luteola* TVnu 172, an accession with a very high level of inhibitory activity. After affinity chromatography on Procion Red Sepharose CL-6B, the purified protein represents less than 0.5% of the total protein loaded on the column. The recovered fractions, eluted with increased ionic strength, were pooled, dialysed and concentrated by ultrafiltration on Amicon YM1 membrane. The specific activity at this step of purification increased about 30 times compared with the ethanol fraction. SDS–PAGE analysis showed that this bound Red Sepharose fraction, active against α-amylase from *C maculatus* larvae, contained four bands corresponding to 23, 22, 11 and 5 kDa (Fig 3). These data are in agreement with literature references showing that proteinaceous α-amylase inhibitors found in cereals and leguminosae are either a trimer or a tetramer of identical polypeptides or different polypeptides, which can be resolved by SDS–PAGE into different bands.[18,23,31,41,43] The purification and better characterization of the Red Sepharose adsorbed proteins are necessary to elucidate which family of inhibitors the ones from cowpea may be classified in.

These results concerning α-amylase inhibitory activity of *Vigna* seeds are correlated with the resistance/susceptibility characteristics of samples analysed. Therefore, the same results are in good agreement with the hypothesis that infestation by the cowpea weevil proceeds normally in the cowpeas until the larva reaches the cotyledons. Here the larva is exposed to the α-amylase inhibitor for the first time and its development rapidly ceases without significant damage to seeds (large cavities, seed weight losses and a concomitant loss in seed viability).[24]

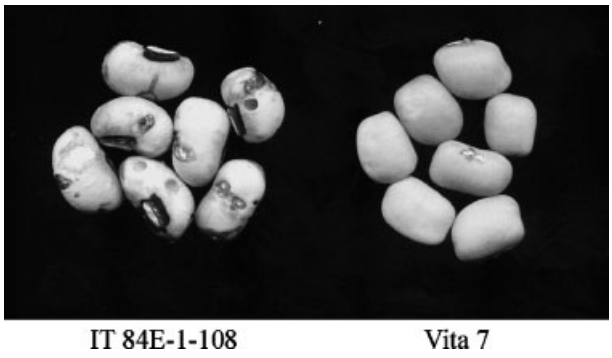

Figure 4. Image of cowpea weevil infestation of two cultivated accessions of *Vigna*: IT 84E-1-108 cowpea seeds show severe damage caused by bruchids and Vita 7 cowpea seeds show no damage.

Table 2. Antinutritional factors of cowpea seeds

Cowpea accession	Proanthocyanidin content (mg g^{-1} dry seed coat)	α-Amylase inhibitory activity (I_{50})[a]
Vita 7	32.0	nd[b]
IT 84E-1-108	2.4	26.0

[a] I_{50}, µg of protein that gives 50% inhibition of insect α-amylase.
[b] nd, not detectable.

However, Fig 4 shows two accessions of stored cowpea seeds, both classified as susceptible accessions, showing a different degree of bruchid damage in storage: IT 84E-1-108 exhibited an elevated degree of infestation (about 30%), while Vita 7 did not show damage caused by cowpea weevil larvae. Surprisingly, no α-amylase inhibitory activity was found in cotyledons of Vita 7 seeds, while IT 84E-1-108 exhibited a moderate level of inhibitory activity (Table 2). In contrast, the seed coat tannin content was found to be 13 times higher in undamaged Vita 7 seeds than IT 84E-1-108 infested seeds. These latter results support the hypothesis that, if bruchids infest cowpea when the grain is stored after harvest, seed coat tannins are effectively involved in the biochemical defence mechanisms, which can deter, poison or starve bruchid larvae that feed on cowpea seeds.

CONCLUSIONS

In conclusion, the abundant literature references concerning the resistance mechanisms of plant tissues against insects strongly suggest that the ecological relationship between insects and plant tissues is a complex one with physical as well as chemical interactions.[44] As far as the mechanism of seed resistance against bruchids is concerned, many strategies are used by seeds to protect themselves against insects: (i) the seed may be too hard for newly hatched larva to penetrate; (ii) the seed may physically be too small or with an inconvenient shape for the larva to reach full size; (iii) the seed may contain

too little food to support the larva; and (iv) the seed may contain toxins or other substances in the cotyledons or its enveloping seed coat that inhibit the larval development.[45] There are conditions when these latter chemical defences can be made inadequate, so bruchids are able to infest seeds. Firstly, many plants suffer reductions in defence compounds during their developmental cycle. Secondly, just as plants evolve defences, their predators evolve tools to evade those defence mechanisms.[41] For example, it has been recently reported that a chemical mechanism is used by insects to overcome protein denaturing compounds, such as tannins. In this connection Konno *et al*[46] observed that some Lepidoptera larvae secrete a large amount of free glycine in digestive juice to counter the protein denaturing activity of host plant tannins. As far as the second chemical defence considered in this paper is concerned, α-amylase inhibitor forms a complex with some α-amylases and, in this manner, is supposed to play a role in plant defence against insects. However, in nature, some bruchids can feed on plants producing α-amylase inhibitors because they possess a serine protease able to cleave some kinds of α-amylase inhibitors.[47,48]

Notwithstanding, the experiments reported here suggest that both seed coat proanthocyanidins and cotyledon α-amylase inhibitor must be considered in the biochemical basis of bruchid resistance in cowpea seeds: both chemical defences could be part of a resistance management strategy, combining a set of insecticidal substances to achieve an adequate bruchid resistance.

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