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CONDENSED TANNIN LEVELS AND RESISTANCE OF GROUNDNUTS (*ARACHIS HYPOGAEA*) AGAINST *APHIS CRACCIVORA*

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Key Word Index—*Arachis hypogaea*; Leguminosae; petiole sap; condensed tannins; proanthocyanidins; *Aphis craccivora*; aphid fecundity; insect resistance.

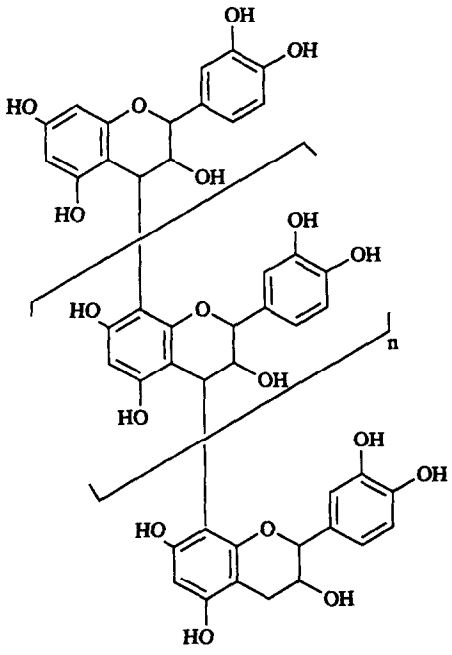
Abstract—A strong negative relationship was found between the concentrations of procyanidin, a condensed tannin, in the leaf bud petioles of seven genotypes of groundnut (*Arachis hypogaea*) and fecundity of the aphid *Aphis craccivora* on the same genotypes. Genotype EC 36892 contained the highest amount of procyanidin per weight of fresh petiole (*ca* 0.7%) and aphids feeding on this genotype produced significantly fewer offspring than aphids reared on genotypes with low procyanidin levels. It is proposed that testing for high procyanidin concentrations may provide plant breeders with a quick and relatively simple method to screen new groundnut genotypes for resistance against *Aphis craccivora*.

INTRODUCTION

The groundnut (*Arachis hypogaea* L.) is an important oil, food and fodder crop which plays a significant role in the agricultural economy of countries in the semi-arid tropics. Over the last two decades the yield of this crop has suffered in sub-Sahelian West Africa due to attack by Groundnut Rosette disease [1]. This is caused by a viral complex including the Groundnut Rosette Virus (GRV) and the Groundnut Rosette Assistor Virus (GRAV), which is transmitted in a persistent manner by the aphid, *Aphis craccivora* (Koch) [2, 3]. Resistance of the host plant to the aphid vector helps to control the disease under field conditions, as has been demonstrated for the groundnut genotype EC 36892 (ICG 5240) [4]. The aim of this study was to identify the nature of aphid resistance in groundnut genotype EC 36892 in order to facilitate the differentiation between aphid susceptible and resistant progeny in breeding programmes.

Electronic monitoring of stylet probing by aphids indicated that the main resistance mechanism in EC 36892 could be associated with the phloem sieve elements [5]. The time spent by aphids probing in this tissue was reduced by 50% when compared to those on a susceptible genotype, TMV 2 [5]. Thus in order to understand the factors responsible for groundnut resistance to *A. craccivora*, attempts were made to collect and analyse groundnut phloem sap. Initial analyses indicated that EC 36892 petiole sap contained nearly twice as much procyanidin (a proanthocyanidin = condensed tannin) per unit weight as the sap from TMV 2. As procyanidin isolated from *Sorghum bicolor* Moench has been shown to be the most active feeding deterrent against biotype C of the aphid *Schizaphis graminum* (Rondani) [6], its presence in groundnut warranted further investigation.

In the present study the procyanidin concentration in seven groundnut genotypes was determined and compared with the fecundity of *A. craccivora* reared on the same genotypes. From the results the possible role and significance of procyanidin in groundnut aphid resistance is discussed.



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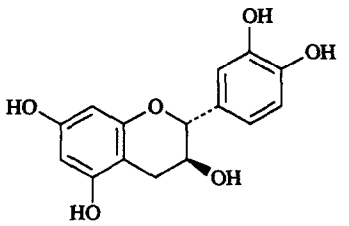

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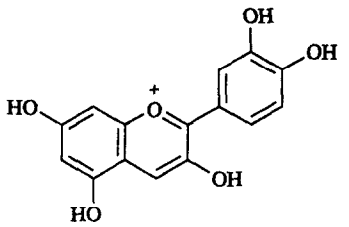

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RESULTS

Detection and identification of procyanidin and precursor in wound sap and EDTA exudate samples

Attempts were made to obtain groundnut phloem sap by means of the aphid stylet cutting technique [7]. Although sap exuded from 10 severed aphid stylets on both EC 36892 and TMV 2, exudation stopped after the production of only one droplet. The amounts collected were in the order of 0.001 μl, and these were too small for chemical analysis. Therefore, for both EC 36892 and TMV 2, 'wound' sap samples containing mesophyll, xylem and phloem sap were collected instead, and the EDTA technique [8] was used in an attempt to collect phloem sap exudates. Both types of samples were examined by means of PC and HPLC.

PC of the wound sap and EDTA exudate was severely hindered because of the presence of a large amount of a substance which was not moved from the origin by the solvents used (BAW and 15% aq. HOAc). This substance was colourless in visible light. However, it appeared as a dark absorbing spot under UV light and after a few days it turned pink-brown in daylight. Any amino acids and sugars in the samples appeared to be held back by this compound and did not separate well. Heating the sample with dilute hydrochloric acid resulted in the formation of a deep red coloration, which indicated that a proanthocyanidin (=condensed tannin) was present. TLC of the resulting red fraction showed the presence of cyanidin, so that the proanthocyanidin was tentatively identified as a procyanidin polymer (**1**). This procyanidin was present in the leaf petiole wound sap and EDTA exudate of both EC 36892 and TMV2, but the concentration in petioles of mature leaves was approximately 1.8 times greater in EC 36892 compared to TMV 2.

To see whether any other UV-absorbing substances were present in the petiole sap, a wound sap sample and an EDTA exudate of EC 36892 were purified by means of PPC to remove the procyanidin and then examined by HPLC. No clear peaks could be distinguished on the HPLC chromatograms of the purified EDTA exudate, but several on that of purified wound sap sample. One of these peaks had the same retention time (R_t 5.9 min) and a similar UV spectrum (λ_{max} 280 nm) as (+)-catechin, a procyanidin precursor. However, the concentration was low (*ca* 0.03% per weight of fresh petiole.)

Comparison of exudation and extraction methods of procyanidin from groundnut plants

To make an accurate comparison between the procyanidin levels in the various groundnut genotypes, the best method of exuding or extracting the tannin from the petioles had to be established.

The average amounts of procyanidin exuded from the petioles of whole leaves of EC 36892 into EDTA (method 1) and the amounts extracted into EDTA and 50% acetone from cut up petioles (methods 2 and 3) varied from an average of 81–88 μg per petiole or from 5.4–6.2 μg mg^{-1} petiole (Table 1). The results of these methods overlapped and were not significantly different. The extraction methods using petioles without leaves were considered more suitable for comparative studies as the results could be expressed per weight of fresh petiole. Leaves contained little or no procyanidin (results not shown).

Extraction into 50% acetone has some advantages over EDTA extracts since the acetone solution mixes with the butanol reagent for the procyanidin determinations and a two-layer system and emulsion do not form. However, most of the procyanidin measurements were performed at high ambient temperatures (*ca* 30°) and evaporation of the acetone during the 4-hr extraction, despite the closed vials, increased the variability of the results. Thus the method chosen to compare the procyanidin concentrations was extraction into EDTA of cut petioles (method 2).

Procyanidin concentrations in EDTA exudates of seven groundnut genotypes.

The procyanidin levels of seven groundnut genotypes were compared. Apical petioles of closed leaf buds (nine samples of three petioles each) were used for one series of determinations and those of just unfolding leaves (10 samples of two petioles) for the second series (young apical petioles are the organs favoured by this aphid [9]). Extracts were made in EDTA as described in method 2. A revised version of the method described by Porter [10]

Table 1. Amount of proanthocyanidin measured in petioles of EC 36892 using different exudation/extraction methods (mean ±s.d.)

	Amount of proanthocyanidin (μg)	
Exudation/extraction method	per petiole	per mg of petiole
EDTA exudate of whole leaves	82 (5)	—
EDTA exudate of cut up petioles	81 (19)	6.2
50% acetone extract of cut up petioles	88 (34)	5.4

was applied for the quantitative determination of procyanidin. The average amounts of procyanidin per weight of petiole measured for each genotype are shown in Table 2. Substantial variation (see standard deviations) in procyanidin concentration was found within each genotype and an even greater variance between genotypes. The resistant genotype, EC 36892, showed the highest mean value of procyanidin in the petioles of closed buds (7.34 $\mu g\ mg^{-1}$ or *ca* 0.7% per fr. wt of petiole) and this was approximately three to eight times higher than the mean concentrations in the petioles of TMV 2, FDRS 10 and GBPRS 15. Genotype GBPRS 312 had the second highest and GBPRS 66 and ICGS 44 intermediate concentrations. In all genotypes except GBPRS 312, petioles of opening leaf buds had lower procyanidin levels than those of closed bud petioles (see Table 2).

Aphid fecundity on seven groundnut genotypes

Aphid fecundity was determined independently of the procyanidin determinations, but using plants grown at the same time under the same conditions. Fecundity over the first five days of reproduction on the seven groundnut genotypes is shown in Table 3.

Nearly twice as many nymphs were produced on GBPRS 15 and FDRS 10 and 1.7 times as many on GBPRS 66, TMV 2 and IGC 44 as on genotype EC 36892. Apart from aphids on EC 36892, those on GBPRS 312 also had a low fecundity compared with the other five genotypes.

DISCUSSION

The number of offspring of *A. craccivora* produced in the first five days of reproduction was lowest on groundnut genotypes with the highest proanthocyanidin content in their petiole exudates and *vice versa*. This negative relationship between aphid fecundity and condensed tannin concentration was strongest with genotype EC 36892 which is known to be resistant to the aphid. The results of the fecundity measurements also indicate that aphid resistance is present in GBPRS 312. Honeydew production by aphids feeding on artificial diets can be reduced by the addition of procyanidin [6] and high levels of this substance could therefore be responsible for the chemical resistance of groundnuts to aphids by acting as a feeding deterrent with secondary effects on fecundity.

Several types of secondary plant metabolites have been strongly linked with aphid resistance, including hydroxamic acids in wheat [11, 12] and the indole alkaloid, gramine, in barley [13], but little is known about their mode of action. This is also the case with condensed tannins and their interactions with aphids and other invertebrate herbivores.

Tannins are substances carrying a multiplicity of phenolic groups, often of high molecular weight (up to 20 000) which have the capacity to bind proteins [14]. They can be divided into two groups, the condensed and the hydrolysable tannins. Condensed tannins are polymers of flavan-3-ols [e.g. catechin = cyanidol-3 (2)] which on treatment with alcoholic mineral acids are converted to red coloured anthocyanidin pigments [principally cyanidin (3) and delphinidin], hence the name proanthocyanidins. Tannins are well known to lower the growth rate of insect herbivores; until recently it was believed that this was caused by complexation to proteins from the food and in the insect's gut. It has been shown now that the formation of insoluble tannin-protein complexes in the intestinal tract of insects is highly unlikely because of the detergent activity of the gut fluids [15]. A literature survey revealed that only tannin concentrations which surpass normal dietary levels tend to deter insects from feeding [15]. Thus high concentration rather than the presence *per se* of condensed tannins seems to be the important factor. This is consistent with the results presented here in that only genotypes EC 36892 and GBPRS 312, with their higher concentrations of procyanidin, offer significant levels of aphid resistance.

Aphids have stylet mouthparts which penetrate plant tissues, and they feed primarily from the phloem sieve elements [16]. Electronic monitoring of aphid feeding [5] indicated that aphid stylets remained in the sieve elements of the susceptible genotype TMV 2 for significantly longer periods than in the resistant EC 36892, thus suggesting that a deterrent was present in the sieve elements of EC 36892. Proanthocyanidins have been reported as being in the phloem and bark of trees [17, 18],

Table 2. Proanthocyanidin concentrations in EDTA leaf exudates from apical petioles of seven groundnut genotypes (mean ± s.d.)

Genotype	Proanthocyanidin concentrations (μg/mg fresh petiole) of closed leaf bud ($n=9$)	of opening leaf bud ($n=10$)
GBPRS 15	0.85 (0.35) a	0.33 (0.07) a
FDRS 10	1.70 (0.71) ab	1.34 (0.33) ab
TMV2	2.29 (1.09) ab	0.96 (0.41) ab
GBPRS 66	2.90 (0.82) abc	2.73 (0.92) bc
ICGS 44	3.44 (1.20) bc	2.99 (1.12) bc
GBPRS 312	4.08 (0.87) bc	5.60 (2.10) c
EC 36892	7.34 (2.59) c	6.99 (1.11) c
	$H=46.3$	$H=60.2$

Kruskal–Wallis analysis of variance by ranks. Values within a column followed by the same letter are not significantly different, non-parametric Tukey-type multiple comparisons ($p<0.01$).

Table 3. Number of nymphs produced in first five days of reproduction by *A. craccivora* on seven groundnut genotypes (mean ± s.e.)

Genotype	*N*	Number of nymphs produced
GBPRS 15	15	38.5 (2.48) a
FDRS 10	15	37.3 (2.67) a
GBPRS 66	15	33.7 (2.46) ab
TMV 2	15	33.6 (2.61) ab
ICGS 44	15	32.1 (1.53) ab
GBPRS 312	14	25.1 (1.43) bc
EC 36892	14	19.5 (3.20) c

One way analysis of variance $F=7.73$ (*df* 6.96). Values followed by the same letter are not significantly different, Tukey test ($p<0.05$).

but their specific location in the different cells types, i.e. phloem parenchyma, sieve elements, companion cells has not been described. The location of procyanidin storage in the groundnut petiole tissue is presently being determined by histological examination. Also the effect of procyanidin on the feeding behaviour of *A. craccivora* is under investigation by adding different concentrations to artificial diets.

A precursor of procyanidin, catechin, has been reported to act as a feeding deterrent to the rose aphid, *Macrosiphum rosae* (L.) [19]. Therefore, the negative relationship between procyanidin concentration and aphid fecundity in groundnut could reflect an indirect mode of action involving catechin. This substance was found in sap pressed from rose petioles [19], but in the present study it was not found in groundnut EDTA exudates and in only very small quantities in the wound sap. We therefore conclude it is unlikely to be a phloem feeding deterrent or a useful marker in detecting aphid resistance in groundnut genotypes.

The variability in concentrations of procyanidin found within genotypes and between slightly different growth stages of the same genotypes indicates that conditions for chemical screening must be standardised carefully and include the growth conditions and petiole age as well as the extraction technique. If these conditions are met the technique described above should provide plant breeders with a quick and relatively simple method for screening new groundnut genotypes for aphid resistance.

EXPERIMENTAL

Plant material. The groundnut genotypes chosen for simultaneous chemical analysis and aphid fecundity were EC 36892 (=ICG 5240), TMV 2 (ICG 221), GBPRS 15 (ICGV 86030), GBPRS 66 (ICGV 86535), GBPRS 312, FDRS 10 and ICGS 44. Of these genotypes, EC 36892 was known to be resistant to *A. craccivora*, and TMV 2, GBPRS 15 and GBPRS 66 susceptible [9]. The response of GBPRS 312, FDRS 10, and ICGS 44 was unknown.

All plants used for the aphid performance and chemical analysis experiments were examined 20–27 days after sowing. The seeds had been treated for 24 hr with Etheral, a dormancy breaking chemical, and were sown in 150 mm pots containing an alfisol from the ICRISAT experimental farm. Plants were grown in an air-conditioned greenhouse, temp 26° ±1°, with natural illumination, dawn at 06:10, dusk at 18:20.

Plant organs used for chemical examination. Petioles of unfolded apical leaves (*ca* 10–30 mm) of the main shoot of genotypes EC 36892 and TMV 2 were used to collect samples from cut aphid stylets, wound sap and EDTA exudates. The latter two were employed for HPLC and PC analysis and wound sap for procyanidin purification.

Younger petioles from the main apical shoot were used for procyanidin determinations of the seven groundnut genotypes. Two slightly different stages of development were studied: petioles of closed leaf buds (*ca* 3–5 mm long), and petioles of just unfolding leaf buds (*ca* 5–10 mm long) (Fig. 1).

The aphid stylet technique for obtaining phloem sap. Stylets of aphids which were feeding from groundnut plants and had produced one of more droplets of honeydew, were cut by positioning a fine etched tungsten needle attached to a microcautery unit against the aphid's rostrum without disturbing the insect. Ten aphid stylets on both EC 36892 and TMV 2 were cut and in each case a drop of sap was observed at the cut surface. This was collected in a micropipette.

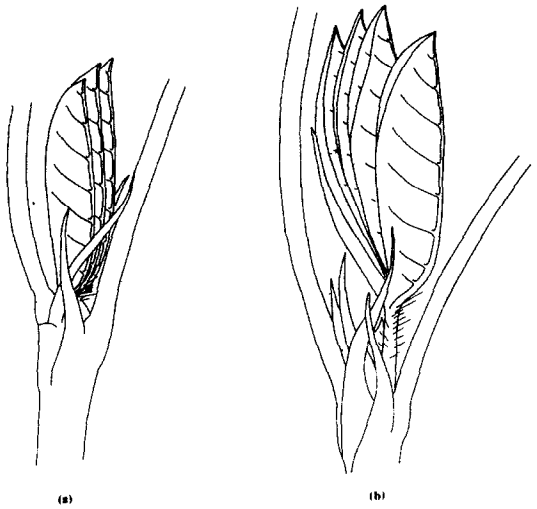

Fig. 1. Leaf buds of groundnut (a) closed and (b) unfolding.

Collection of groundnut 'wound' sap. The apical petiole was severed from a groundnut plant with a sharp razor blade and sap exuding from the wound was collected in a glass capillary. Several microlitres of sap per wound were obtained in this way. The capillary tubes were weighed before and after sap collection and the sap was then blown into a glass vial. The vials contained 1.5 ml of 50% aq. Me_2CO for procyanidin measurements and 1 ml 80% MeOH for HPLC examination. For PC, either pure wound sap or wound sap mixed with 1 ml 80% MeOH was used.

Detection and tentative identification of proanthocyanidins. To 1 ml of wound sap samples in test tubes, 3 ml of 2 M HCl was added. The tubes were heated for 30 min at 100° in a water bath. After cooling, the now red-coloured solution was thoroughly mixed with 0.5 ml of amyl alcohol. After separation of two layers, the concd, red top layer was pipetted off and evapd to dryness. The residue was dissolved in a drop of methanolic HCl and examined by means of TLC (cellulose) for anthocyanidins using three different solvents and cyanidin and delphinidin as standards.

PC and PPC of wound sap and EDTA exudate samples. Spots of wound sap and concd EDTA exudates were applied to Whatman No 1 paper. After running in BAW (*n*-BuOH–HOAc–H_2O, 4:1:5, upper layer) or 15% aq. HOAc, the papers were examined in longwave UV light (366 nm).

In order to examine samples for compounds other than proanthocyanidins, the latter had to be removed since they interfered with the determinations. This was done by means of PPC. The samples were applied as streaks onto quarter sheets of Whatman no 3 MM paper and the chromatograms were run in BAW. After development, the UV light absorbing band of procyanidin which stayed at the origin was cut off and the remainder of the paper eluted with 80% MeOH. The eluate was concd and filtered before examination by means of HPLC.

HPLC of wound sap EDTA exudates. Aliquots of the various samples were examined by means of reverse phase HPLC (C_{18} phenyl column, 30 cm, 3.9 mm i.d.). A linear gradient of H_2O (solvent A) and MeOH–H_2O–HOAc, 18:1:1 (solvent B) was used for elution. The gradient started at 75% A and 25% B and changed to 0% A and 100% B over 23 min. The flow rate was 1.0 ml min^{-1}. A sample of 0.1% (+)-catechin was run as a standard (R_t 5.9 min; λ_{max} 280 nm).

Comparison of exudation and extraction methods. Three differ-

ent methods of obtaining procyanidin were compared, using apical petioles of genotype EC 36892 (*ca* 10–30 mm long):
(1) Petioles with leaves were placed in 1.5 ml of a 20 mM solution of disodium EDTA dihydrate in H_2O (pH adjusted to 7 with 2M KOH) immediately after cutting from the plant and left to exude for 4 hr. This method is similar to the EDTA phloem exudation method as described in ref. [8].
(2) Petioles with leaves were severed from the plant. Holding the leaflets, the petiole was cut with scissors into 3 mm pieces which were immediately immersed without handling in 1.5 ml of EDTA (20 mM pH 7) in a glass vial. Only petiole material was extracted; the leaflets were discarded after the petiole pieces had been cut off. The vial was closed with a lid and the petioles were left to extract for 4 hr. The vials were weighted before and after the addition of the petioles to calculate the fr. wt of the plant material.
(3) Method 3 was the same as that in (2) but instead of using EDTA, the petiole material was immersed in 50% aq. Me_2CO, a good extraction solvent for proanthocyanidins [10].

For all three methods, times longer than 4 hr gave smaller readings, perhaps because of enzyme activity, so extractions were not carried out overnight.

Measurement of proanthocyanidins. The method used for the quantitative determination of proanthocyanidins was based on that described in ref. [10]. Into a thick walled test tube, containing 3 ml *n*-BuOH–conc. HCl (19:1), a 1 ml aliquot of wound sap, EDTA exudate or Me_2CO extract was pipetted. To the Me_2CO extracts but not to the EDTA exudates (for reasons see below), 0.1 ml of a 2% soln of $NH_4Fe(SO_4)_2 . 12 H_2O$ in 2 ml HCl was added. The sample and reagents(s) were mixed using a whirler mixer. EDTA samples and the BuOH reagent separated out into an aq lower layer and organic upper layer, whereas the 50% Me_2CO sample and BuOH reagent were miscible. After mixing the test tubes were suspended in a hot water bath at 85° for 40 min with a marble placed on top of each tube to minimise evapn. Concurrently 1 ml aliquots of 6 different concs of purified groundnut procyanidin dissolved in the same solvent as used for the samples (EDTA or Me_2CO) and 1 ml of the pure solvent were processed in the same way as the petiole samples to produce a standard curve and blank respectively. After heating the solns were left to cool for 30 min or until the solns cleared. The extinction of the BuOH (which was carefully pipetted off to avoid clouding when there were two layers) was recorded at 550 nm (λ_{max}) on a spectrophotometer. The quantities of proanthocyanidin in the samples could be determined by comparison with the extinctions of known concentrations of procyanidin. Sometimes persistent emulsions would form between the BuOH and aq. layers and samples in which the emulsion did not clear were discarded as they gave far higher readings in the spectrophotometer than clear samples of the same colour intensity.

Since metals can form complexes with procyanidin and consequently metal traces can alter the results of the above method, it is advocated to add a surplus of ferric ions in the form of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ to the reaction mixture in order to increase the reproducibility of the method [20]. However, when this reagent was added to the samples in EDTA, the BuOH layer remained colourless after heating whereas the proanthocyanidin precipitated as an orange brown substance in the aq. layer. Thus the ferric reagent could not be used in the case of the EDTA sample. However, since EDTA chelates metal ions it may play a similar role as the ferric reagent in increasing the reliability of the method.

Purification of the groundnut petiole proanthocyanidin. Purified proanthocyanidin from petiole wound sap of EC 36892 was used to produce standard curves for spectrophotometric measurements. Petioles were removed from plants with a razor blade and after a few min sap was collected by means of a capillary tube from the wounded surfaces of the plants. This liquid was blown into glass vials stored at −20°. Aliquots of this sap were defrosted, mixed with an equal amount of MeOH and the mixture adsorbed onto Sephadex LH-20 in 50% aq. MeOH in a small glass column or Pasteur pipette. Impurities were removed from the sample by eluting with 50% MeOH. The procyanidin was eluted from the Sephadex column with Me_2CO–H_2O (7:3) [8] and 2 ml frs were collected. A few drops of each fr. was heated with 1 ml of the BuOH–HCl reagent and frs which were positive for procyanidin (red coloration with the reagent) were combined. The Me_2CO was removed from these combined frs by means of a rotary evaporator at 20° and the remaining aqueous solution was freeze-dried to yield the purified proanthocyanidin as a pinkish powder.

Determination of aphid performance. 20 apterous virginoparae were confined singly in clip cages [21] on a closed leaf bud of a groundnut apical growing point (Fig. 1). These aphids were left for 24 hr to produce nymphs and then removed using a fine paintbrush together with all but one first instar nymph (day 1). The cages were checked daily to record development and the number of offspring produced in the first 5 days of reproductive life. Winged aphids (alates) were not included in the experiment, and the cages containing the insects were moved to new closed leaf buds every 5 days.

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