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CHANGES IN GROWTH, PROTEINS AND FREE AMINO ACIDS OF DEVELOPING SEED AND POD OF FENUGREEK

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Key Word Index—*Trigonella foenum graecum*; Leguminosae; fenugreek; maturation; seed; pod wall; growth; proteins; free amino acids; 4-hydroxyisoleucine.

Abstract—The growth and development under field conditions of the pods and seeds of two cvs of *Trigonella foenum graecum* are described. Samples were harvested at different stages of ripeness for the determination of dry matter, protein and free amino acid content. During maturation, reserves of solutes are established in the pod wall before the seeds begin their exponential phase of growth. Later, these reserves disappear, providing about 20% of the seed's requirements for nitrogen. SDS-electrophoresis was used to follow the formation of proteins and it was shown that the synthesis of storage proteins takes place prior to dehydration of the seed. Production soluble nitrogenous compounds precedes protein accumulation. Free amino acids follow the same pattern. 4-Hydroxyisoleucine represents nearly 80% of free amino acid of dry seeds. The concentration does not decrease in the later stages of maturation of the seed but this unusual amino acid is absent in the storage proteins of the seeds.

INTRODUCTION

Fenugreek (*Trigonella foenum graecum* L., Papilionaceae, Leguminosae) is an annual leguminous plant whose seeds contain about 30% of protein. The level of lysine in proteins is comparable to the level of lysine in soybean proteins and as determined on rats, it has a good nutritional value [1]. Solubilization and characterization of these proteins by electrophoresis have been studied elsewhere [2]. However, fenugreek contains an 'unusual' free amino acid identified as 4-hydroxyisoleucine [3].

We have no knowledge of any study of changes of fenugreek proteins and free amino acids during pod formation and seed maturation, and the aim of this work has been to investigate how nitrogenous constituents are formed during development of fenugreek seeds in relation to the adjacent tissue, the pod wall. Moreover, we have studied the changes in free amino acids and focused upon the variation of 4-hydroxyisoleucine which had been proposed as a possible precursor for the characteristic flavouring component of fenugreek [4].

RESULTS AND DISCUSSION

Development of pod and seeds

The changes in fr. wt and dry wt of the different parts of the whole pod: seeds and pod wall are shown in Figs 1 and 2 for the two cvs. Fr. wt of pods increased rapidly from 7–14 days after flowering (DAF) to about 35–42 DAF. Thereafter, as the seeds desiccated, fr. wt declined rapidly from about 42 DAF. The maturation of fenugreek seeds was completed in about two months depending upon atmospheric conditions. Dry wt of the pods increased more slowly than fr. wt and attained final weight 56 DAF for cv Cocipa and 49 DAF for cv Gouka. In the case of both cvs, there was a diminution of dry wt at the final time of maturation.

The changes in the weight of the pod are due, firstly to the increase in the weight of the pod wall (fr. and dry wt) and secondly, and more importantly, to the growth of the

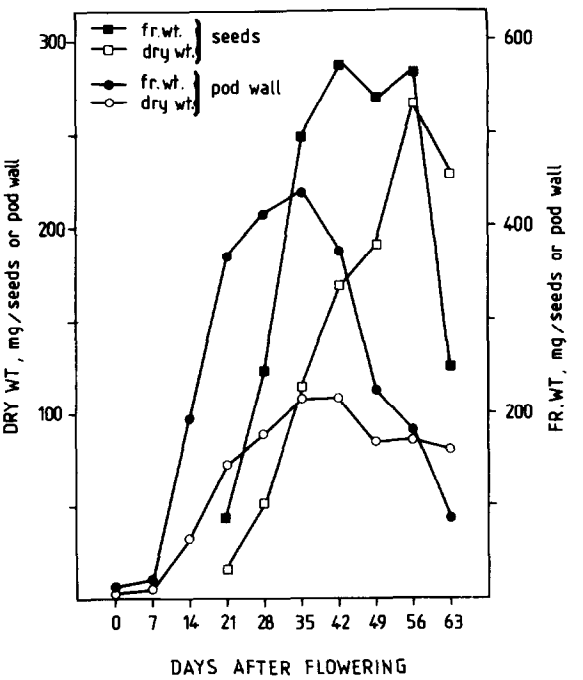


Fig. 1. Development of seeds contained in one pod and pod wall of fenugreek, cv Cocipa at different stages of maturation.

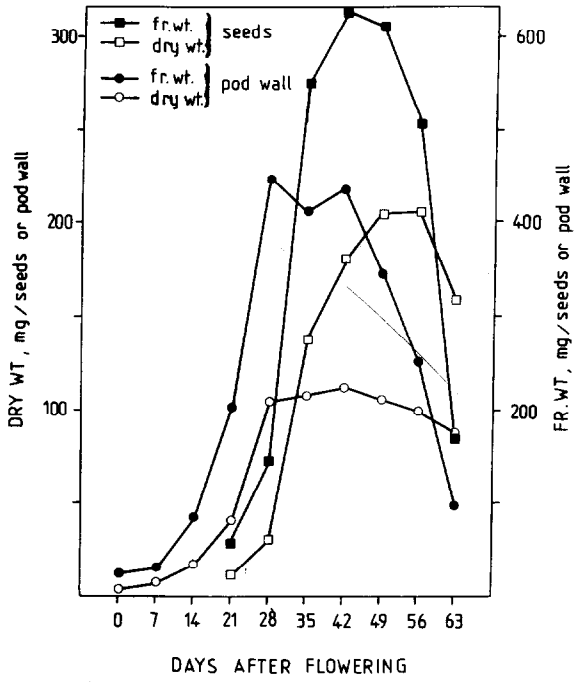


Fig. 2. Development of seeds contained in one pod and pod wall of fenugreek, cv Gouka at different stages of maturation.

seeds. Seed growth begins when the pod wall is still growing as for bean plants sown in autumn [5]. In beans of the spring sowing, there is practically no overlap between the main growth period of the pod and that of the seed [5]. Here, the growth of the pod and the seed is sequential and not concurrent.

The slight diminution of dry wt observed for the pod wall, estimated to 21 at 26% according to the cvs of fenugreek does not explain the great increase of weight of the seeds. However, an estimate of the greatest possible transfer to seeds through mobilization of reserves of the pod wall was 12–15%.

What is the origin of metabolites for the seeds? Willmer and Johnston [6] have shown that in the case of fenugreek, certain enzymes involved in CO_2 fixation and metabolism, namely phosphoenolpyruvate carboxylase, NADP malic enzyme and NADP malate dehydrogenase (NADP, MDH) have an activity consistently higher in the non-leaf tissues (outer layers of green pod and green seeds). These authors think that the pod tissues have neither typical C-4-pathway photosynthesis (obvious lack of dimorphic chloroplasts type 'Krantz'; inability to maintain a CO_2 compensation point always > 240 ppm), nor Crassulacean acid metabolism, but that the increased levels of certain enzyme activities are present to refix and recycle respired CO_2, thereby minimising the losses of CO_2, making the organ more efficient.

There is some evidence also that the pod tissues of *Phaseolus vulgaris* [7], the reproductive structures of *Glycine max* [8], of *Vicia faba* [6] are also involved in reassimilating respired CO_2. In leguminous plants, the photosynthesis contribution of the reproductive organs is low compared to that of the leaves [5, 9]. In contrast, the reproductive organs of cereals have a relatively high net photosynthesis contributing to at least 50% of the photosynthetate for grain-filling (refs in [8]).

Evolution in protein nitrogen of pod and seeds

The variation of protein nitrogen as mg per pod wall and mg per seeds contained in one pod is shown in Figs 3 and 4. The accumulation of protein nitrogen in pod wall

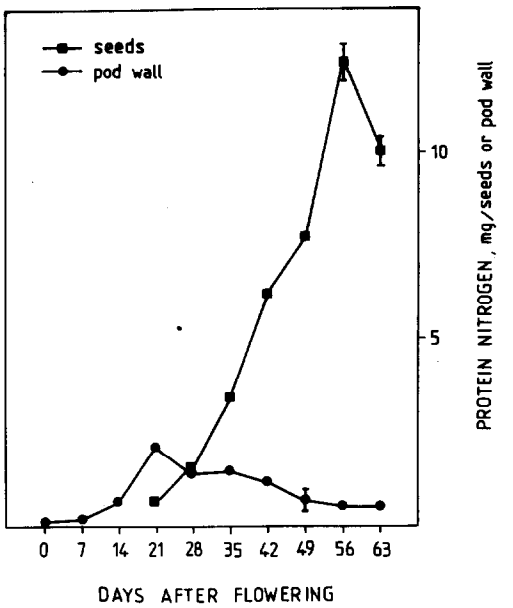


Fig. 3. Protein nitrogen of seeds and pod wall at different stages of maturation, cv Cocipa. Bars represent $t \times$ s.e. ($P \leqslant 0.05$) and where absent are within the dimensions of the symbols.

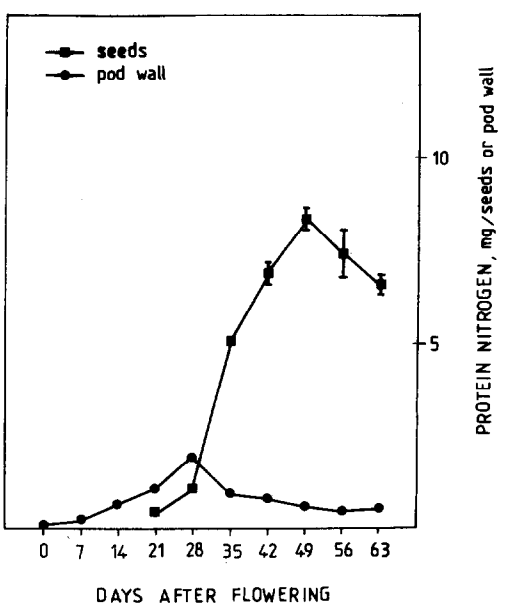


Fig. 4. Protein nitrogen of seeds and pod wall at different stages of maturation, cv Gouka. Bars represent $t \times$ s.e. ($P \leqslant 0.05$) and where absent are within the dimensions of the symbols.

and seeds is sequential. The protein nitrogen content of the seeds increases sharply from 28 DAF, a stage where the protein nitrogen of the pod wall has already declined.

It is possible that the pod wall supplies one part of the nitrogen to the seeds. Losses of 1.6 mg and 1.4 mg of protein nitrogen from a pod wall of cv Cocipa or Gouka respectively occur between 21–28 DAF seed maturity. They represent over half of the nitrogen accumulated during the earlier life of the pod wall.

The estimate of maximum possible transfer to seeds through mobilization of protein nitrogen reserves of the pod wall, was 20–27% for fenugreek. By comparison, the transmission of 24% of total seed nitrogen from the pod wall could be calculated for field pea *Pisum arvense* [10] and 13% [11] or 28% [12] for garden pea *Pisum sativum*. In the case of *Lupinus albus*, phloem is suggested to supply on the average 98% of the carbon, 89% of the nitrogen and 40% of the water entering the fruit from the parent plant. The remaining nitrogen and water are assumed to be supplied by xylem and from the fruits photosynthetic activity [13].

A continuous flow into the seed from the vegetative plant must take place until the funiculum desiccates and the seed becomes nutritionally a closed system [14]. The PAGE-SDS analysis shows that fenugreek proteins found in mature seeds may be distinguished from the 35th DAF. Practically all of them are already present in the immature seed 49 DAF. From that date, changes occurring until maturity concern mostly the intensity of protein bands. However, especially in the case of the cv Cocipa (Fig. 5), some interesting qualitative and quantitative modifications of the protein composition could be observed between the 35th and 42nd as well as between the 42nd and 49th DAF. In these periods, some polypeptides seem to appear then disappear in the immature seed. However, from the 49th to the 56th DAF a range of proteins, more

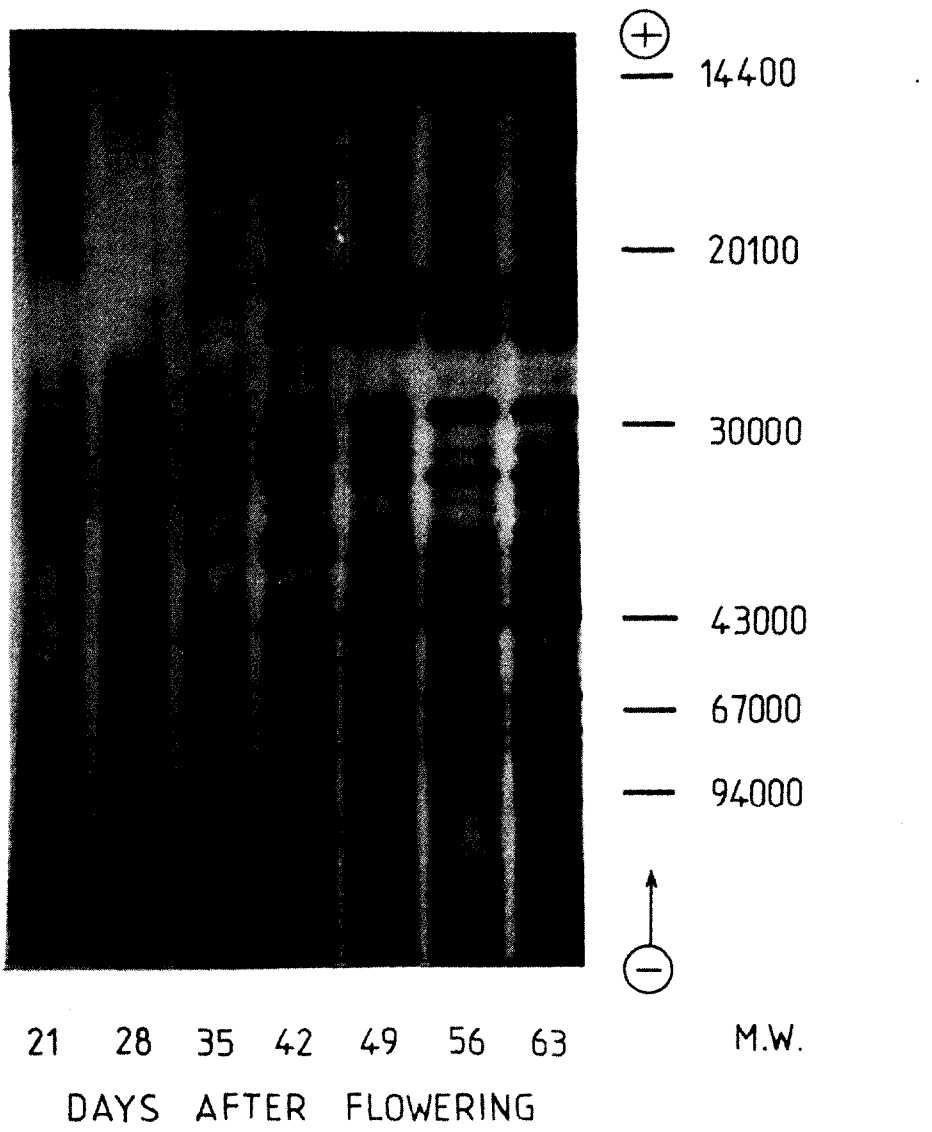


Fig. 5. Distribution of protein components by MW at different stages of seed development, cv Cocipa (vertical PAGE-SDS of reduced proteins).

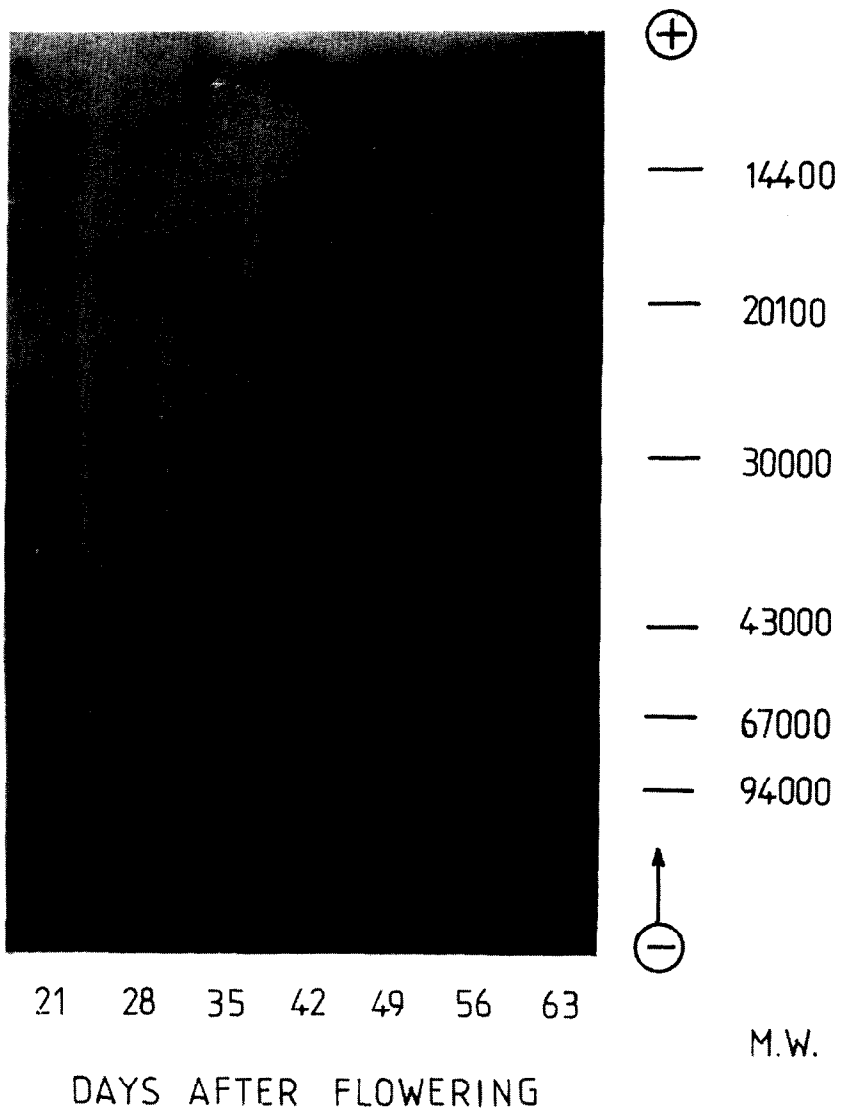


Fig. 6. Distribution of protein components by MW at different stages of seed development, cv Gouka (vertical PAGE-SDS of reduced proteins).

typical of the mature seed, has been established, and relatively few further changes occur until the seed becomes dry. Protein bands were not detected in the samples taken before 35 DAF, although Lowry's protein reaction was positive. This can be attributed to the presence of small peptides in the early stage of maturation as has been already reported for others leguminous seeds [10, 15].

In general according to our results, the development of proteins during maturation is very similar for the two cultivars that we studied. It is interesting to notice the large number of protein bands and the marked heterogeneity in their MW which ranges from around 10 000 to over 100 000. It is known that seeds constitute a sink where storage proteins are deposited in protein bodies [16], that way seeds gain a contribution from the rest of the plant.

Changes in the pod and seeds nitrogen soluble content and free amino acids; case of 4-hydroxyisoleucine

The soluble nitrogen reaches a maximum 28 DAF for the pod wall and 35 DAF for the seeds. Then, as the seed is developing, soluble nitrogen progressively decreases in these two parts of the fruit. The amount of free amino acids follows the same pattern as soluble nitrogen. The concentrations of the various amino acids changed during the course of development. For all stages of maturation, 4-hydroxyisoleucine is the principal free amino acid but with a variable percentage. This is at a maximum at full maturity where 4-hydroxyisoleucine represents 80% of the total free amino acid for the two cultivars.

Contrary to other amino acids which show a sharp decline with ripeness, the 4-hydroxyisoleucine remains at

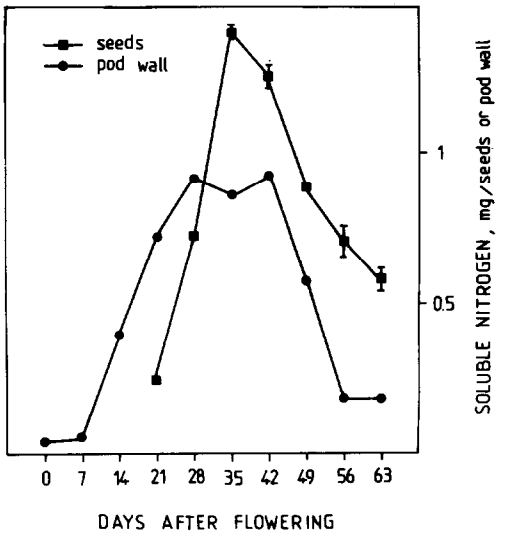


Fig. 7. Soluble nitrogen of seeds and pod wall at different stages of maturation. Bars represent $t \times$ s.e. ($P \leqslant 0.05$) and where absent are within the dimensions of the symbols.

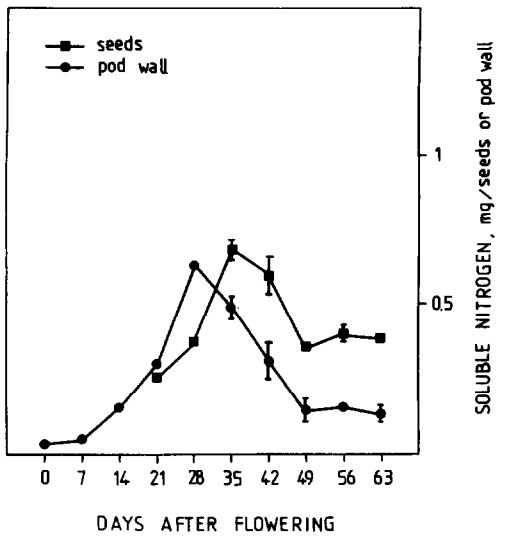


Fig. 8. Soluble nitrogen of seeds and pod wall at different stages of maturation. Bars represent $t \times$ s.e. ($P \leqslant 0.05$) and where absent are within the dimensions of the symbols.

a high level. This result indicates that this amino acid does not have an important role in protein biosynthesis. This hypothesis has been verified by the amino acid composition after acid hydrolysis of seed proteins before and after free amino acid extraction (−AAL). The 4-hydroxyisoleucine is absent from defatted seed −AAL and it is not included in the storage proteins of the seeds. Moreover, using ion exchange chromatography of the extract, we have verified that the 4-hydroxyisoleucine is not found in the soluble sugars with steroidal saponins and organic acids fractions. However, we have not found steroidal saponins in the free amino acid fraction. Accordingly, our results do not confirm the presence of fenugreekine, the steroidal sapogenin peptide ester previously isolated [17] but not mentioned since in any publication. We have not detected canavanine though its presence has been described previously [18]. It is possible that this compound is eluted at the same time as 4-hydroxyisoleucine in its lactone form.

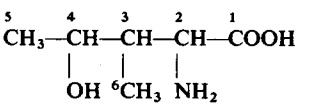
EXPERIMENTAL

Plant material. Two cvs were used: Cocipa and Gouka planted in Autumn in two separate years in field conditions at Montpellier, France. The experimental plot contained about 20 000 plants and samples were collected when plants were *ca* 50 cm high. Flowers and pods of the same morphology and maturity were harvested in large numbers. Seeds were removed from the pod wall on the 28th day after flowering (DAF). After counting and weighing, samples were freeze dried for 4–5 days. Before analysis, the material was ground into a powder in order to pass through a 70 mesh sieve.

Isolation and identification of 4-hydroxyisoleucine. The powdered and defatted seeds (100 g) were extracted × 6 with 70% EtOH. After concn under vacuum, the amino acids were fixed on an Amberlite IR 120 H^+ form (column 36 × 2 cm) then eluted with 1 M NH_3. The amino acid fraction was lyophilized and chromatographed on silica gel 60, 70–230 mesh (column 40 × 2.5 cm). The elution was performed with a solvent: $CHCl_3$–MeOH–H_2O (10:5:1). The fractions containing 4-hydroxyisoleucine (determination by TLC) were pooled; crystallization and purification gave 0.6 g of 4-hydroxyisoleucine which was identical with an authentic sample provided by Sir Leslie Fowden using TLC, amino acid analyser, NMR, SM.

TLC. This was carried out on silica gel G plates with *n*-BuOH–HOAc–H_2O (4:1:1) as solvent then heated at 110° for 10 min, followed by spraying ninhydrin (0.1% in Me_2CO), R_f = 0.2.

^1H NMR spectroscopy. This was performed at 90 MHz using a soln of 4-hydroxyisoleucine in D_2O; trimethylsilylpropane sulfonic acid was used as internal standard. Resonances were observed at δ 0.95 and 1.25 (doublets, attributable respectively to protons of Me groups C-6 and C-5), 1.85 (broad multiplet, proton of C-3) and 3.85 (multiplet, including a doublet due to a proton of C-2 and a multiplet due to proton of C-4).



EIMS. 70 eV, *m/z* (rel. int.): 148 $[M+H]^+$ (5), 102 $[M+H-CO_2H_2]^+$ (32), 74 $[M+H-74]^+$ (92), 58 $[M+H-74-16]^+$ (100).

Molecular beam solid analysis, fast atom bombardment [19] (glycerol, 3 KeV): 148 $[M+H]^+$ (100), 130 $[M+H-H_2O]^+$ (35), 102 $[M+H-CO_2H_2]^+$ (30), 74 $[M+H-74]^+$ (56).

FAB/MS/MS. By the procedure of ref. [20] on ion *m/z* 148:148 $[M+H]^+$ (86), 130 $[M+H-H_2O]^+$ (100), 102 $[M+H-CO_2H_2]^+$ (19), 74 $[M+H-74]^+$ (51).

Free amino acid extraction and analysis. Powdered samples (5–10 g) were extracted in a Soxhlet apparatus successively with *n*-hexane and 70% *iso*-PrOH–H_2O. Aq. extract was fractionated on Amberlite IR 120, H^+ form (5 × 1 cm) then eluted with 1 M NH_3. The amino acid analyses were performed with a Durrum model D 500 automatic analyser. A Durrum DC 4A chromatographic resin was used (8 ± 1 μm, 8% cross linking); it was packed in a stainless steel column with an inside diameter of 1.75 mm and a length of 48 cm. It was necessary to execute two analyses. One with three Na citrate buffers for classical separation and another

Table 1. Levels of free amino acids in flowers and seeds during maturation (n mol/flower or seed), cv Cocipa

Amino acid	Flower	Seed (days after flowering) 28	35	42	49	63
Aspartic acid	12.9	15.4	18.6	33.9	19.3	8.4
Threonine	20.7	35.3	98.7	106.7	47.9	5.9
Serine	24.8	33.0	77.5	94.1	54.3	4.2
Asparagine	197.5	114.5	215.2	209.7	84.4	14.1
Glutamic acid	5.3	16.0	24.1	20.2	10.2	22.1
Glutamine	9.5	27.0	20.0	15.5	3.4	tr.
Proline	90.7	154.8	97.3	118.1	90.9	14.1
Glycine	und.	und.	und.	und.	und.	und.
Alanine	19.0	160.1	236.4	320.5	181.6	tr.
Cystine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Valine	41.7	145.3	228.1	154.4	68.5	5.6
Methionine	tr.	tr.	tr.	tr.	tr.	tr.
Isoleucine	5.7	49.0	109.9	88.3	38.3	4.1
Leucine	5.1	33.3	132.2	124.9	47.6	4.0
Tyrosine	2.8	11.6	27.4	34.3	5.6	10.9
Phenylalanine	3.9	79.2	158.5	71.0	23.9	9.1
γ-Amino-*n*-butyric acid	41.9	165.5	54.8	76.0	387.4	7.2
Histidine	tr.	tr.	tr.	tr.	tr.	tr.
Lysine	1.0	1.8	10.8	8.6	4.7	0.9
Ethanolamine	5.9	34.4	62.4	90.2	44.5	7.4
Arginine	0.8	88.6	24.4	21.9	15.6	9.4
4-Hydroxyisoleucine	464.1	579.6	1106.1	941.2	664.4	726.3
Number of flowers or seeds used for the extraction	5774	5793	6318	5952	7049	6617

und, Undetermined; tr, traces; n.d., non-detectable.

with only one Li citrate buffer to separate glutamine and asparagine from threonine, serine and glumatic acid. The first analysis was performed in 105 min with a buffer soln at a flow rate of 8.6 ml/hr. The successive pH values of three buffers were 3.25 ± 0.02 4.20 ± 0.02, 7.90 ± 0.02; the Na concns were respectively 0.2 N, 0.2 N and 1.1 N. The initial temp of the column was 51.2°, the second temp used was 61.3°, the change was made 38 min 30 sec from the start. The second analysis was performed in 75 min with a buffer soln flow rate of 6.2 ml/hr, the pH value was 2.85 and the Li concn 0.24 N. The column temp was 46.2°.

Amino acids were detected by a ninhydrin reagent using dimethyl sulfoxide as the solvent [21]. The calibration sample was obtained from Pierce Chemical Company and the 4-hydroxyisoleucine standard was isolated with a procedure described above. The operating and integration system was provided by a PDP 8 digital computer. The experimental error of these analyses procedure gave average coefficient of variation from 1.4% to 2.8%. In the case of the free amino acids analyses the 4-hydroxyisoleucine was detected 26 min 50 sec from start between glycine and alanine peaks. However, often the very important amount of 4-hydroxyisoleucine coincided with the glycine peak.

Amino acid composition of the seed and the residue obtained after defatted and iso-PrOH *extraction (defatted seed-AAL).* Samples were hydrolysed with 6 N HCl under nitrogen atmosphere in sealed tubes during 24 hr at 110°. Immediately after, the hydrolysate was vacuum-dried and removed with 2.20 pH Na citrate buffer. The 4-hydroxyisoleucine was detected in its lactone form, 65 min 20 sec from the start, between the histidine and lysine peaks. The calibration for 4-hydroxyisoleucine was performed by hydrolysis in the conditions described for 4-hydroxyisoleucine standard isolated by us.

Soluble nitrogen and total nitrogen. The standard microKjeldahl method was used for the determination of soluble and total nitrogen both in *iso*-PrOH extract and in freeze-dried samples. In this study, protein nitrogen is defined as the difference between the total nitrogen and soluble nitrogen. Values given in the graph represent the mean of four determinations with its standard error of the mean. Statistical analyses were performed at the 5% level of probability.

Extraction and electrophoresis of proteins. The defatted samples (500 mg) were extracted with 10 ml 0.12 M Tris–boric acid (pH 8.9) containing 0.1% SDS by vigorously shaking for 10 min. The suspensions were centrifuged at 25 000 *g* for 20 min and the pellet was re-extracted. SDS-acrylamide gel electrophoresis was performed as described in ref. [22]. Protein samples were treated with a soln containing 2% SDS and 5% mercaptoethanol in 0.05 M Tris–HCl pH 6.8 for 3 min at 97°. 50 μl were applied to the gels slabs of 17.5% acrylamide. Running buffer was 0.025 M Tris–HCl, 0.19 M glycine, pH 8.3 with 0.1% SDS. Pyronine was used as a tracer. Proteins were stained according to ref. [23]. Gels were put for 16 hr into a soln of TCA 12% containing 0.02% of Coomassie Brilliant Blue R 250. Gels were destained in H_2O. Phosphorylase b (MW: 94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100) and α-lactalbumin (14 400) were used as markers in order to evaluate the MW of the proteins.

Table 2. Levels of free amino acids in flowers and seeds during maturation (n mol/flower or seed), cv. Gouka

Amino acid	Flower	Seed (days after flowering) 28	35	42	49	63
Aspartic acid	8.6	8.2	19.8	26.1	6.0	9.2
Threonine	9.8	9.9	34.4	17.2	5.2	3.4
Serine	10.6	16.7	15.8	11.0	6.7	4.2
Asparagine	71.6	31.9	68.1	53.5	5.8	11.9
Glutamic acid	1.9	9.5	30.6	20.2	6.9	13.0
Glutamine	3.3	9.6	14.7	4.4	tr.	tr.
Proline	1.8	68.9	116.4	51.2	28.0	13.9
Glycine	und.	und.	und.	und.	und.	und.
Alanine	10.9	49.5	75.7	59.2	17.5	tr.
Cystine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Valine	3.3	10.2	58.4	35.1	5.1	3.4
Methionine	tr.	tr.	tr.	tr.	tr.	tr.
Isoleucine	2.7	10.3	28.3	12.7	2.7	2.4
Leucine	2.3	7.5	26.8	9.8	2.7	2.7
Tyrosine	1.9	3.8	10.8	4.2	1.2	6.8
Phenylalanine	2.1	7.2	56.8	8.5	5.3	10.4
γ-Amino-*n*-butyric acid	13.5	88.4	92.0	55.4	22.4	5.8
Histidine	tr.	tr.	tr.	tr.	tr.	tr.
Lysine	0.4	1.0	7.4	4.3	0.5	1.1
Ethanolamine	6.1	16.2	41.6	50.4	20.3	7.7
Arginine	2.7	5.2	27.5	13.3	2.6	13.2
4-Hydroxyisoleucine	306.4	305.7	1036.8	794.7	485.8	568.7
Number of flowers or seeds used for the extraction	2389	2370	2772	2860	2448	930

und, Undetermined; tr, traces; n.d., non detectable.

Table 3. Amino acid composition of mature seed proteins before and after AAL* extraction (g/16 g N)

Amino acid	cv Cocipa		cv Gouka	
	Whole mature seed	Defatted seed −AAL*	Whole mature seed	Defatted seed −AAL*
Aspartic acid	11.0	11.6	10.5	10.7
Threonine	3.4	3.2	3.3	3.3
Serine	5.1	4.7	4.9	4.7
Glutamic acid	17.5	18.6	16.5	16.9
Proline	4.6	3.2	4.2	3.7
Glycine	4.7	4.2	4.7	4.5
Alanine	3.6	3.1	3.8	3.9
Valine	3.5	3.5	3.5	3.5
Cystine	1.4	1.3	1.4	1.7
Methionine	0.9	0.7	1.0	0.8
Isoleucine	4.6	4.3	3.9	4.4
Leucine	7.1	6.8	6.2	6.8
Tyrosine	2.9	2.3	2.8	2.4
Phenylalanine	4.2	4.2	3.8	4.0
Histidine	2.3	2.7	2.1	1.9
Lysine	5.9	5.7	6.6	6.0
Arginine	10.0	8.6	8.8	8.9
Tryptophan	0.7	1.0	0.7	1.0
4-Hydroxyisoleucine	2.5	0.0	3.6	0.0

*AAL, Free amino acid.

Determination of steroid saponins. The steroid saponins content was determined by a method described previously [24].

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