Proximate composition, amino acid content and haemagglutinating and trypsin-inhibiting activities of some Brazilian *Vigna unguiculata* (L) Walp cultivars

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Abstract: Seeds of Brazilian *Vigna unguiculata* (L) Walp cultivars (EPACE 10, EPACE 11, Pitiuba, TVu 1888, IPA 206 and Olho de Ovelha) were analysed to establish their proximate composition, amino acid content and presence of antinutritional and/or toxic factors. The seed protein, carbohydrate and oil contents ranged from 195 to 261 g kg^{-1} dry matter, from 678 to 761 g kg^{-1} dry matter and from 12 to 36 g kg^{-1} dry matter respectively. EPACE 10, EPACE 11, Pitiuba, TVu 1888, IPA 206 and Olho de Ovelha cultivars are rich in glutamin/glutamic acid, asparagin/aspartic acid and phenylalanine + tyrosine. The essential amino acid profile compared with the FAO/WHO/UNU scoring pattern requirements for different age groups showed that these seeds have methionine + cysteine as the first limiting amino acid for 2–5-year-old children. However, only Pitiuba, IPA 206 and Olho de Ovelha are deficient in methionine + cysteine for 10–12-year-old children. The contents of threonine, valine, isoleucine, leucine and methionine + cysteine of all cultivars were lower than those of hen egg. Haemagglutinating activity measured against rabbit erythrocytes was found to be present in the six cultivars, but only after the red cells were treated with proteolytic enzymes. All cultivars displayed protease inhibitor activity which varied from about 12.0 to 30.6 g trypsin inhibited per kg flour. Urease and toxic activities were not detected in any of the studied cultivars.

Keywords: *Vigna unguiculata*; seed nutrient composition; amino acid content; antinutritional factors

INTRODUCTION

The species *Vigna unguiculata* (L) Walp, commonly called cowpea, is an important grain legume crop which provides the major source of dietary protein, calories and vitamins for a large segment of the population in sub-Saharan Africa, Brazil and India, thus alleviating malnutrition.[1,2] Unfortunately, in most of these areas there are severe seed yield losses due to drought stress[3] and reduction in seed quality and utilisation because they are attacked by a wide range of insect pests, particularly during storage,[4,5] reducing food supply. The main objectives of the Brazilian improvement programme have been to develop varieties of cowpea tolerant to environmental adversities through a variety of methods such as crosses between or within local and introduced cultivars, pedigree selection, cultivar competition, genealogical selection, hybridisation, bulk and back-cross procedures, recurrent selection and single-pod descent. As a result of these efforts, hundreds of cowpea lines were released, a few of them improved in some traits and thus recommended for cultivation.[6–8] Nevertheless, information on the chemical composition of seeds from these cultivars is scarce. Like most other grain legumes, cowpea contains antinutritional compounds such as trypsin inhibitors, lectins and tannins which decrease protein digestibility and quality.[9] In this work, seeds of six cultivated lines of cowpea sown in north-east Brazil were analysed for their protein content, amino acid composition and presence of toxic and/or antinutritional substances.

MATERIAL AND METHODS

Seeds and reagents

Mature seeds of four varieties of improved cowpea

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(EPACE 10, EPACE 11, Pitiuba and TVu 1888) were obtained from the Agronomy School at Universidade Federal do Ceara, Ceara and two (IPA 206 and Olho de Ovelha) from Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Pernambuco, Brazil. Bovine serum albumin (96%), coomassie brilliant blue G (98%), Kunitz-type soybean trypsin inhibitor (type I-S), *N*-α-benzoyl-L-arginine *p*-nitroanilide (L-BAPNA), dimethyl sulphoxide (99.9%), phenol (99.5%), bromelain (5–10 units mg^{-1}), papain (10–20 units mg^{-1}), subtilisin (7–15 units mg^{-1}), trypsin (type I) and urease (41H7008, 870 000 units g^{-1}) were purchased from Sigma Chemical Co, St Louis, MO, USA.

Physical assessments

Seed mean weight was calculated from 100 grains from each variety selected randomly. To estimate dimensions, five groups of 10 grains each were selected at random and the length and width were recorded using a sliding calliper.

Proximate analysis

Seeds were analysed for moisture, ash and oil contents according to Triebold.[10] Total nitrogen[11] was used for determination of seed crude protein contents ($N \times 6.25$). Seed carbohydrate contents were calculated by difference.

Amino acid composition

Amino acid analyses were performed after hydrolysis of seed flours with 6 M HCl plus 10 $g l^{-1}$ phenol at 110 °C for 22 h in sealed glass tubes under N_2 atmosphere. HCl and phenol were removed by evaporation and the amino acid compositions were established after chromatography on a Biochrom 20 system (Pharmacia). Tryptophan content was measured colorimetrically.[12]

Aqueous extracts

The whole seeds were ground in a coffee grinder (Moulinex, Super Junior 'S', Dublin, Republic of Ireland) to a fine powder and stored at 5–8 °C until used. The seed meals were suspended in the extracting solution (0.5 M NaCl, pH 7.0) in the proportion of 1.0 g of meal to 10.0 ml of solution. The suspension was maintained under continuous stirring (400 rev min^{-1}, Stuart Scientific (UK) magnetic stirrer) for 4 h at 4 °C and then filtered. The filtrate was centrifuged at $16\,000 \times g$ for 20 min at 4 °C and the clear supernatant was dialysed (cut-off MW 12 000) against the extracting solution.

Protein determination

The protein content in the extracts was determined by the method described by Bradford[13] using bovine serum albumin as standard.

Agglutination assay

Haemagglutinating activity present in the seeds was assessed by serial twofold dilution of the extracts.[14] The extracts were diluted with 0.15 M NaCl in glass tubes and mixed with rabbit red cells (20 mg ml^{-1} suspension prepared in 0.15 M NaCl). The degree of agglutination was monitored visually after the tubes had been left to stand at 37 °C for 30 min and at room temperature (22 ± 3 °C) for an additional 30 min. The results are reported as haemagglutination titre (HU), which is the reciprocal of the highest dilution giving visible agglutination.

Trypsin inhibitor activity

Trypsin inhibitor assay was carried out by a slight modification of the method originally described by Kakade.[15] The meals (0.020 g) were suspended in 1 ml of 0.01 M NaOH. The suspensions were stirred magnetically for 3 h. After this period the mixtures were left for 30 min without stirring and then the supernatants were centrifuged for 5 min at $14\,000 \times g$. After centrifugation, 0.1 ml of the alkaline extract of each cultivar was mixed with 1.6 ml of 0.05 M Tris-HCl, pH 8.2, containing 0.02 M $CaCl_2$, with 0.1 ml of trypsin solution (from a stock solution of 0.4 mg in 10 ml of 0.001 M HCl) plus 0.1 ml of *N*-α-benzoyl-L-arginine *p*-nitroanilide solution (10 mg ml^{-1} in 97% dimethyl sulphoxide plus H_2O, 1:3 v/v). The mixtures were incubated for 45 min at 37 °C and then 0.2 ml of 30% acetic acid solution was added. The absorbance at 410 nm was measured. Activity was expressed as the amount of trypsin inhibited, calculated from a calibration curve using soybean trypsin inhibitor.

Urease assay

Urease assay was carried out by minor modifications of the procedure described by Kaplan.[16] 0.1 ml of urea solution (0.5 M) and 0.7 ml of 20 mg ml^{-1} EDTA buffered with 0.2 M potassium phosphate, pH 6.5, were mixed with 100, 200 or 300 µl of the seed crude extracts. The mixtures were incubated for 15 min at 37 °C and then 1.0 ml of phenol plus sodium nitroprusside solution (62.0 g of phenol with 0.25 g of sodium nitroprusside per litre) and 1.0 ml of sodium hypochlorite plus alkali solution (43.0 ml of 52.5 $g l^{-1}$ hypochlorite with 20.0 g of alkali per litre) were added. The mixtures were incubated for a further 5 min at 37 °C. After that, 7 ml of distilled water was added. The tubes were covered with parafilm and shaken vigorously to mix. The absorbance at 625 nm was measured. The enzyme activity was calculated from a calibration curve using urease.

Acute toxicity assay

Toxic activity was defined as mortality observed in mice within 24 h after intraperitoneal injections of the crude extracts.[17]

Statistical analysis

The results were subjected to a one-way analysis of variance and the significance of differences between

Cultivar	Parameters			
	Weight (g)	Length (cm)	Width (cm)	Tegument colour
EPACE 10	0.20±0.04a	0.89±0.08a	0.69±0.06a	Brown
EPACE 11	0.19±0.02b	0.90±0.06a	0.64±0.05b	Brown
Pitiuba	0.18±0.01c	0.81±0.07b	0.63±0.05b	Brown
TVu 1888	0.13±0.01d	0.67±0.05c	0.53±0.05c	Black
IPA 206	0.22±0.02e	1.05±0.06d	0.82±0.07d	Brown
Olho de Ovelha	0.21±0.02f	1.12±0.06e	0.74±0.05e	Whitish

[a] Values followed by different letters are statistically different ($p \geq 0.05$).

Table 1. Physical characteristics[a] of Brazilian *Vigna unguiculata* cultivars

means was determined by Student's *t*-test ($p < 0.05$) using the Excel program.[18]

RESULTS AND DISCUSSION

Seed mean weight and dimension

In spite of the large difference in seed mean weight between TVu 1888 (0.13 g) and the other cultivars (0.18–0.22 g) assessed in the present study (Table 1.), the values are within the range found for several lines of cultivated cowpea seeds, which varied from 0.06 to 0.24 g,[19,20] and higher than those found for wild *Vigna* spp seeds.[21] The seed lengths and widths varied from 0.67 to 1.12 cm and from 0.53 to 0.82 cm respectively, with Olho de Ovelha and IPA 206 respectively showing the highest values.

Proximate composition

Table 2 shows that the crude protein contents of *V unguiculata* seeds (195–261 g kg^{-1} dry matter) are comparable with those of several other cowpea varieties, which ranged from 210 to 270 g kg^{-1} dry matter.[22–24] They are also close to the values reported for other species belong to the genus *Vigna*, such as *V umbelata* (172–181 g kg^{-1} dry matter),[25] *V campensis* (224 g kg^{-1} dry matter),[24] *V mungo* (238–250 g kg^{-1} dry matter),[26,27] *V radiata* (231 g kg^{-1} dry matter),[27] *V angularis* (227 g kg^{-1} dry matter)[28] and *V minima* (185–257 g kg^{-1} dry matter).[29] The high carbohydrate contents observed (678–761 g kg^{-1} dry matter) must have been overestimated, since fibre was not determined in the present study. The carbohydrate contents of several other cowpea cultivars represent around 637 g kg^{-1} dry matter, and fibre around 47 g kg^{-1} dry matter.[24] Regarding the oil (12–36 g kg^{-1} dry matter) and ash (32–41 g kg^{-1} dry matter) contents, these are similar to other values reported for *Vigna unguiculata* cultivars.[22,24,30] However, it is known that the lipid content varies depending on the cultivar, climate, season and environmental conditions.[31]

Amino acid patterns

The amino acid composition of each seed meal, the minimal requirements established for children (2–5- and 10–12-year-olds)[32] and the amino acid content of hen egg[32] are presented in Table 3. The seeds of the EPACE 10, EPACE 11, Pitiuba, TVu 1888, IPA 206 and Olho de Ovelha cultivars are rich in glutamin/glutamic acid, asparagin/aspartic acid and phenylalanine + tyrosine. Based on the FAO/WHO/UNU[32] scoring pattern, these seeds have methionine + cysteine as the first limiting amino acid for 2–5-year-old children. However, only Pitiuba, IPA 206 and Olho de Ovelha are deficient in methionine + cysteine for 10–12-year-old children. All other essential amino acids meet the children's needs, although the contents of most of them are lower than those for hen egg. It is well known that cystine, cysteine and methionine are destroyed to varying degrees during hydrochloric acid hydrolysis.[33] Often, oxidation of cystine and cysteine to cisteic acid, which is stable under acid hydrolysis conditions, provides more accurate results. Employing the methodology described by Moore,[34] using performic acid oxidation followed by acid hydrolysis, Nnanna and Phillips[35] determined cystine and methionine as cysteic acid and methionine sulphone respectively and observed an increase in the recovery

Cultivar	Components				
	Moisture	Protein[b]	Oil	Carbohydrate[c]	Ash
EPACE 10	143.0±0.2a	237.2±16.2a	22.6±0.8a	698.8±11.3a	41.4±0.1a
EPACE 11	126.4±0.2b	195.0±2.6b	12.2±0.2b	760.8±2.9a	32.0±0.1b
Pitiuba	126.7±0.6b	210.0±5.0c	20.2±0.4c	733.4±5.6b	36.4±0.0c
TVu 1888	120.1±0.1c	210.2±9.2abc	35.8±0.6d	721.1±9.9acd	32.9±0.2d
IPA 206	134.0±0.6d	261.0±9.1ad	24.0±0.9a	677.8±0.6e	37.2±0.2e
Olho de Ovelha	130.8±0.6e	250.1±11.5ad	26.8±0.8e	688.6±0.1f	34.5±0.1f

[a] Means of triplicate analyses. Values followed by different letters are statistically different ($p \geq 0.05$).
[b] N × 6.25.
[c] Calculated by difference.

Table 2. Proximate composition[a] (g kg^{-1} dry matter) of Brazilian *Vigna unguiculata* cultivars

Table 3. Amino acid composition (g kg^{-1} protein) of Brazilian *Vigna unguiculata* cultivars compared with hen egg and FAO/WHO/UNU[32] scoring patterns of amino acid requirements for children

	Cultivars							Children[a]	
Amino acid	*EPACE 10*	*EPACE 11*	*Pitiuba*	*TVu 1888*	*IPA 206*	*Olho de Ovelha*	*Hen egg*	*2–5 years*	*10–12 years*
Essential									
Thr	43.3	39.7	39.3	39.4	39.6	38.9	47	34	28
Val	41.6	41.1	40.6	36.9	44.0	43.4	66	35	25
Ile	48.1	34.4	34.0	36.5	45.8	45.1	54	28	28
Leu	71.8	76.0	76.0	77.7	72.7	73.0	86	66	44
Lys	66.4	70.9	70.5	70.3	69.1	70.2	70	58	44
Phe + Tyr	111.1	112.2	106.4	111.8	105.0	105.0	93	63	22
Met + Cys	23.6	23.9	17.8	22.0	20.3	20.1	57	25	22
Trp	13.6	21.0	24.2	28.6	13.6	12.6	17	11	9
His	38.8	36.4	36.3	36.6	37.2	36.7	22	19	19
Non-essential									
Asx	108.2	118.6	119.3	114.0	108.6	107.2			
Glx	168.6	173.9	180.7	172.0	196.1	196.7			
Ser	44.6	47.6	49.6	53.0	41.0	41.8			
Gly	39.4	40.7	40.2	41.8	39.6	35.6			
Ala	46.0	46.9	47.0	43.5	42.4	41.8			
Arg	85.0	70.8	73.5	73.4	82.0	85.2			
Pro	49.9	45.9	44.7	44.4	46.1	47.1			

[a] Patterns of amino acid requirements for different age groups.

of these amino acids. In our experiments we used 6 M HCl + 10 g l^{-1} phenol to prevent degradation of some amino acids.[33] In this condition, loss of methionine and cysteine may have occurred, but to a lesser extent. Indeed, comparison of our results (Cys + Met, 17.8–23.9 g kg^{-1} protein) with those obtained for other cultivars of *Vigna unguiculata* submitted to performic acid oxidation prior to HCl treatment showed contents of methionine + cysteine (21.4–26.0 g kg^{-1} protein) similar to those found by Nnanna and Phillips.[35] Furthermore, the results presented in this paper are higher than those reported by Olaofe *et al*[23] for other species of *Vigna unguiculata* and for another representative of *Vigna*, *V umbellata*.[36] Nevertheless, amino acid composition data have shown that methionine is the first limiting amino acid for most leguminous seeds.[37]

Antinutritional compounds and toxicity

The presence of lectins is depicted in Table 4. Haemagglutinating activity measured against rabbit erythrocytes was found to be present in the six cultivars, but only after the red cells were treated with proteolytic enzymes. The haemaglutinating activity seems to be exclusively due to the presence of lectin and not promoted by polyphenols, tannins and lipids,[38] since it was fully abolished by heat treatment (92 °C, 5 min) of the cowpea aqueous extracts. Generally, trypsin-treated cells were the most sensitive to agglutination, probably owing to greater exposure of

Table 4. Lectin activity of the crude extracts from Brazilian *Vigna unguiculata* cultivars against enzyme-treated rabbit erythrocytes

	Cultivars					
Lectin activity	*EPACE 10*	*EPACE 11*	*Pitiuba*	*TVu 1888*	*IPA 206*	*Olho de Ovelha*
Total (HU per seed)[a]						
Bromelain	951	0	533	1329	590	920
Papain	898	1886	533	2658	453	856
Subtilisin	951	1886	533	1329	453	1236
Trypsin	1200	1886	1067	5317	820	2573
Specific (HU mg^{-1} protein)[b]						
Bromelain	22643	0	15676	55375	11569	20000
Papain	21381	57152	15676	110750	8882	18609
Subtilisin	22643	57152	15676	55375	8882	26870
Trypsin	28571	57152	31382	221542	16078	55935

[a] Total haemagglutinating activity (HU) was calculated based on the mean seed fresh weight (Table 1).
[b] Specific haemagglutinating activity was taken in relation to the seed crude protein content (Table 2).

Table 5. Trypsin inhibitor activity[a] ($g\,kg^{-1}$ flour) of Brazilian *Vigna unguiculata* cultivars

Cultivar	*Trypsin inhibited*
EPACE 10	15.64 ± 0.38a
EPACE 11	29.26 ± 0.48b
Pitiuba	22.66 ± 0.36c
TVu 1888	30.56 ± 0.54b
IPA 206	16.80 ± 1.14a
Olho de Ovelha	12.00 ± 0.56a

[a] Each value is an average of three determinations. Values followed by different letters are statistically different ($p \geq 0.05$).

the carbohydrate moieties present in the cell membrane for which the lectin has higher affinity. It is well documented that several seed lectins are resistant to proteolysis by gut enzymes and are detrimental to rat health when fed orally, leading to impaired growth and alterations of key organs, particularly hypertrophy of the small intestine.[39–41] The seed trypsin inhibitor activity is shown in Table 5. TVu 1888 and EPACE 11 have similar values that are about double those found for EPACE 10, IPA 206 and Olho de Ovelha. Overall these values are lower than those reported for some cultivars of Brazilian soybeans, which varied from 30.6 to 62.5 g trypsin inhibited per kg flour.[17] Dietary trypsin inhibitors are claimed to be responsible for the poor digestibility of dietary protein by interference with the proper function of trypsin, leading to growth inhibition and pancreatic hypertrophy.[42] In spite of their antinutritional effects, lectins and trypsin inhibitors are often inactivated by appropriate heat treatment. However, excessive heating could lead to damage to proteins and consequent unavailability of amino acids. Furthermore, not all seeds when submitted to heat treatments show improved nutritional quality. For example, rats fed severely heat-processed bean (*Phaseolus vulgaris*) neither gained nor lost weight at the end of a 10 day experimental period.[43] Also, cooked cowpea and pigeon pea (*Cajanus cajan*) showed a tendency to decrease the body weight gain of young rats as cooking time increased, an effect perhaps caused by a decrease in available lysine due to a non-enzymatic browning reaction induced by the high temperature developed in the mass near the wall of the container.[44] Regarding urease activity, it was not detected in any of the six studied cultivars. In the acute toxicity test, *V unguiculata* extract-treated mice did not exhibit signs of toxicity and no mortality was observed up to 3 g protein kg^{-1} mouse body weight within 24 h.

CONCLUSION

The cultivars of cowpea that were analysed in this investigation constitute a valuable protein source which can contribute towards overcoming protein energy malnutrition in the developing countries. However, there is a need to encourage the practice of combining these cultivars with other food sources in order to make up for their various amino acid deficiencies. Although the presence of lectin and trypsin inhibitor in cowpea seeds represents a nutritional disadvantage, they can be eliminated by moist heat treatment or cooking, since they are heat-labile.

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