Grouping of Brazilian accesses of lima beans (*Phaseolus lunatus* L.) according to SDS-PAGE patterns and morphological characters

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ABSTRACT. Lima beans (*Phaseolus lunatus)* are well adapted to the tropical lowlands, especially to low fertility soils of the most humid areas. To verify the difference among accesses of *Phaseolus lunatus*, twenty four accesses were characterised in relation to flower and hypocotil colour, seed size and colour pattern. They were also submitted to SDS-PAGE. The accesses of *Phaseolus lunatus* presented variations in electrophorectical patterns and were grouped in eleven protein profiles. Using morphological and eletrophorectical patterns, they were grouped in 20 different categories. Morphological and protein patterns have been reliable to separate Lima beans accesses.

Key words: Lima beans, protein electrophoresis, *Phaseolus lunatus,* grouping.

RESUMO. Agrupamento dos acesso de feijão fava (*Phaseolus lunatus*) segundo os padrões SDS-PAGE e caracteres morfológicos. Feijões fava (*Phaseolus lunatus)* são bem adaptados a solos tropicais, especialmente a solos inférteis de áreas úmidas. Para verificar a diferença entre acessos de *Phaseolus lunatus*, 24 acessos foram caracterizados quanto à coloração de flor e de hipocótilo, ao tamanho e padrão de cores em sementes e ao padrão obtido em análise de SDS-PAGE. Os acessos de *Phaseolus lunatus* apresentaram variações nos padrões eletroforéticos e foram agrupados em 11 grupos. Usando os caracteres morfológicos e eletroforéticos, os acessos foram agrupados em 20 diferentes categorias. Os caracteres morfológicos e os padrões de proteínas foram adequados para separar os acessos de feijão fava.

Palavras-chave: feijão fava, eletroforese de proteínas, *Phaseolus lunatus,* grupamento.

Introduction

Lima beans (*Phaseolus lunatus)* are well adapted to the tropical lowlands, especially to low fertility soils of the most humid areas. There are recognised cultigroups based in form and weight of 100 seeds, which are known as "papas" (small seeded, 35 to 50 g for 100 seeds), "sieva" (medium and plane seeded, 50 to 70 g) and "big lima" (big seeded, from 70 to 110 g) (Castineiras *et al*., 1991). Lima bean is a widespread culture throughout tropical America. Geneticists have been improving this crop, so there are more precocious cultivars, with more vigorous maturation, resistant to diseases, pests and without toxins.

Although the lima bean is largely consumed, it is one of the few vegetables that can contain toxic amounts of cyanogenic glycosides in almost all varieties. White types are usually free, but some blacks types are very bitter and possess enough amount of toxins to be considered harmful; they have to be long cooked and the cooking-water, discarded (Cobley, 1957).

Due to the great variability of *Phaseolus lunatus* and the need of producing new forms genetically improved, the correct identification of those varieties becomes necessary. The development of chemical procedures to characterise cultivars improved the identification methods. Protein electrophoresis constitutes a powerful tool for qualitative and quantitative analysis of chemical compositions in the seeds and in seedling parts accepted by many international institutions as differentiation tools among cultivars (Cooke, 1995; Alfenas, 1998). Seed protein electrophoresis seems to be an useful technique for soybean cultivar identification inside of a wide category (Orf *et al*., 1980; Pinto *et al*., 1995). Using a combination of

number of bands and location of the bands, the SDS-PAGE technique (Sodium Dodecil Sulphate - Polyacrylamide Gel Electrophoresis) demonstrated a satisfactory discrimination of seven cultivars of *Phaseolus vulgaris* (Hussain *et al*., 1986). Proteins are the most direct expression of the genes, and the main advantage of its use, in comparison to the classic descriptive techniques, it is the direct determination of a organism genetic expression, independent of environmental influences, considering that the patterns of the seed proteins are highly stable (Sanches-Yelamo *et al*., 1992).

Electrophorectical pattern of the seed's total proteins, revealed by SDS-PAGE, has been producing valid evidences for the discussion of taxonomy and evolutionary problems. In Leguminosae, the patterns of SDS-PAGE bands of the seed's total proteins has been useful for species, subspecies and at variety levels in *Trifolium* (Badr, 1995), *Lotus* and *Vicia* (Pryzybylska and Zimniak-Pryzybylska, 1997), *Phaseolus* beans (Gomes *et al*., 1984; Romero-Andreas and Bliss, 1985; Romero-Andreas *et al*., 1986; Osborn *et al*., 1986; Gepts *et al.,* 1986; Hussain *et al*., 1986; Gepts, 1990; Castineiras *et al*., 1991; Echeverrigaray *et al., 1*993; Driedger *et al*., 1994), mungbean (Tomooka *et al*., 1990) and *Arachis* (Lanham *et al*., 1994). The use of total proteins electrophoresis technique can aid in the characterisation of soybean cultivar, allowing their separation in groups (Orf *et al*., 1980; Pinto *et al.,* 1995).

In this work, the main objective was to characterise different accesses of *Phaseolus lunatus*, from the Cenargen collection, using the SDS-PAGE technique.

Material and methods

Seed material

Seeds of 24 accesses of *Phaseolus lunatus* (Table 1), donated by the Cenargen-Embrapa (National Center of Genetic Resources), were used. The seeds were classified according to the following variables: seed background colour, coloration pattern and size. The variables hipocotyl colour and flower colour were also considered.

Protein extraction

Six seeds in each access had the testa removed and were ground to thin flour in a ball mill (Tecnal) for two minutes. The resulting flour was conditioned in aluminium foil packing and stored at-18ºC. Flour samples (250 mg of each access) were placed in essay tubes and the extraction buffer was added (1:10 w:v) prepared as follows: 25.6 mL of distilled water, 8 mL of 0.625 M Tris-HCl pH 6.8, 12.8 mL of glycerol, 12.8 mL of SDS 10%, 1.6 mL of bromophenol blue 0.05%; and 3.2 mL of β-mercaptoethanol, and put to rest for 1h. After that, the tubes were boiled during 3 min. The material was centrifuged at 10000 rpm for 10 minutes and the supernatants were conditioned in Eppendorfs and stored in freezer (-18ºC). Protein quantification was performed according to Sedmak and Grossberg (1977). The concentration of proteins was calculated by the formula: P = (A595. F)/V, where: P = protein $\mu g \mu l^{-1}$, A595 = absorbance at 595 nm, F = factor (166) obtained by regression analysis and V = volume (5 μl).

Table 1. Name, access number and origin of used seeds of *Phaseolus lunatus*

Access number	Name	Origin
GL022	Fava	Emater/Município de Diamantino-MT
GL025	Valls-5976	CENARGEN
GL026	Fava amarela	Miraporanga/Dist. de Uberlândia-MG
GL027	Fava pataca	Miraporanga/Dist. de Uberlândia-MG
GL042	Fava preta	CENARGEN(003069)
GL049	Fava	CENARGEN(G25148)
GL052	Fava sieva	CENARGEN
GL061	Fava	CENARGEN(003166)
GL062	Fava	CENARGEN(003361)
GL072	Fava chumbinho	CENARGEN(002895)
GL090	Fava manteiga	CENARGEN(002887)
GL098	Fava vermelhona	CENARGEN(Nº529)
GL0115	Fava	Ceará
GL0125	Fava	Rio Pardo de Minas-MG
GL0159	Fava	Jacinto-MG
GL0178	Fava pernambucana	Córrego da Plaina/Ceres-GO
GL0184	Fava grande rajada	Verdolândia/Barra Alta-GO
GL0186	Fava branca achatada	Santa Amália/Inhumas-GO
GL0188	Fava vermelha	Capim Puba/Itapuranga-GO
GL0194	Fava branca achatada	Munic. De Firminópolis-GO
GL0342	Fava branquinha	Sitio Solar do Ninho-GO
GL0355	Fava Jackson Wonder	Sem informação
GL0476	Fava violeta chata	Goiânia-GO
GL0491	Fava grande rajada	Montenegro-RS

Electrophoresis

Electrophoresis was carried out according to Laemli (1970), in a system composed by a running gel containing 20% acrylamide-bisacrylamide (30:0.8), pH 8.8, and a stacking gel with 5% acrylamide-bisacrylamide, pH 6.8, at 30 mA for 1:30 h at 4ºC. Gels were 1,5 mm thick. Aliquots of 30 µg of protein were loaded per well. The running buffer was composed of Tris (25 mM) :Glycine (38 mM) : SDS (0.7 mM), pH 8.8. The gels were fixed with isopropanol:acetic acid:water (4:1:5) for 30 min and stained in the same solution containing 2% Coomassie Blue R250 until the protein bands appeared. If the coloration was too dark, the gel was destained in 10% acetic acid.

The apparent molecular weights determination was accomplished through the Low range markers

(6.5 to 66 KDa; Sigma) composed by these proteins: bovine albumin (66), ovalbumin (45), gliceraldeid-3-phosphate dehydrogenase (36), carbonic anhidrase (29), tripsinogen (24), tripsin inhibitor (20.1) and lactalbumin (14.4). The markers were ran parallel to the seed extracts and molecular weights calculated according to Weber and Osborn (1969).

The evaluation was made by observing the presence/absence of bands of the same apparent molecular weight, and a binary matrix was built using also the morphological characters. The grouping of each species was made by PC-ORD (2.0 MJM Software Design), using the nearest neighbour group linkage procedure.

Results

Protein mobility in the gel allowed the separation of twenty different proteins (Table 2), and this separation was enough to propose eleven protein patterns based in the absence of specific bands. The protein profiles were as follows: pattern I, absence of the bands 1(78 KDa), 9 (50 KDa) and 12(45,8 KDa); pattern II, absence of the band 1; III, 1, 2(74 KDa) and 9; IV, 4(62 KDa) 9 and 12; V, 1, 7(56 KDa) 9 and 12; VI, 2 and 9; VII 9 and 12; VIII, 1 and 9; IX, 9; X, 8(54 KDa) and 12; and XI, 1, 9, 12, 19(26 KDa) and 20(23 KDa).

Table 2. Eletrophorectical mobility and molecular weight of each band detected in the 24 accesses of *Phaseolus lunatus*

Band	Eletrophorectical mobility	Molecular Weight (KDa)
1	0,08	78
2	0,09	74
3	0,115	70
4	0,163	62
5	0,181	60
6	0,198	58
7	0,214	56
8	0,230	54
9	0,248	50,8
10	0,263	49
11	0,280	47
12	0,297	45,8
13	0,330	42
14	0,363	38
15	0,409	35,5
16	0,445	32
17	0,462	30
18	0,495	28
19	0,528	26
20	0,578	23

The morphological analysis of all accesses could split then in several categories and increase the number of classes, as shown in Table 3. Using flower or hypocotil colour we can divide them in two types; using the seed size, we can divide it into five classes. However, the protein pattern can group all the 24 accesses in just eleven categories of higher similarity, as storage seed proteins are well conserved during evolution.

It is observed that the accesses GL0491, GL0178, GL0115, GL0184, GL0098, GL0186, GL0027, GL0022, GL0042 and GL0342 did not present the band of molecular weight 78 KDa, while the band of 74 KDa is absent in 4 accesses: GL0188, GL0061, GL0342, GL0042 and GL0027 (Figure 1 and 2). Only the accesses GL0178 and GL0090 exhibited the band of 50,8 KDa. The accesses GL0159, GL0491, GL0025, GL0194, GL0476, GL0090, GL0072, GL0125, GL0184, GL0098 and GL0022 did not show the band of molecular weight 45,8 KDa (Figure 1 and 2). Figure 3 is exhibiting the results of grouping accesses using electrophoregrams and morphological markers. Accesses G0491, G0194, G0355, GL0090 and G0022 are the more divergent materials among the tested accesses, and G0049 and G0052 could not be differentiated by group analysis and are very closely related with GL0062. GL0025 and GL0026 were also highly related, maintaining a close relation with GL0072. GL0159 and GL0476 were nearly related, as the GL0115 and GL0178. The others accesses were more scarcely related among themselves.

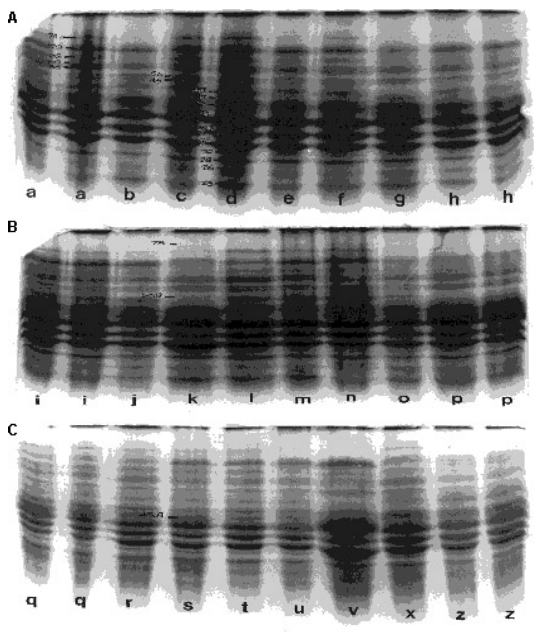

Figure 1. Total soluble protein electrophoresis of *Phaseolus lunatus* accesses. 1A - a - GL0159; b - GL0491; c - GL0025; d - GL0062; e - GL0194; f - GL0026; g - GL0178; h - GL0115; 1B - i - GL0476; j - GL0186; k - GL0090; l - GL0072; m - GL0125; n - GL0052; o - GL0184; p - GL0098; 1C - q - GL0355; r - GL0049; s - GL0188; t - GL0061; u - GL0042; v - GL0342; x - GL0027; z - GL0022

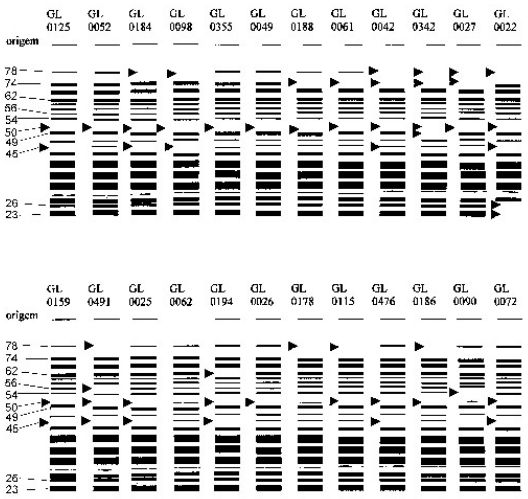

Figure 2. Diagram of electrophoretic pattern of *Phaseolus lunatus* accesses. Arrows show absence of bands. Molecular weights (kDa). The defined patterns were: pattern I, absence of the bands 1, 9 and 12; pattern II, absence of the band 1; III, 1, 2 and 9; IV, 4, 9 and 12; V, 1, 7 9 and 12; VI, 2 and 9; VII 9 and 12; VIII, 1 and 9; IX, 9; X, 8 and 12; and XI, 1, 9, 12, 19 and 20

Discussion

The use of protein as biochemical or molecular markers have been reported for several cultures, an it is accepted by many international organisations to differentiate cultivars (Cooke, 1995). The differentiation of different types of phaseolin is widely used to separate races (Gepts *et al*., 1986; Gepts, 1990; Maciel *et al*. 1999), to identify progenitors and their offspring (Gomes *et al*., 1984) and to cultivar differentiation (Hussain *et al*., 1986). Maciel *et al*. (1999), working with different accesses of *Phaseolus* from *Rio Grande do Sul*, observed a narrow relationship between seed weight and protein pattern (phaseolin). It was observed in the present study that the electrophorectic pattern VI (absence of the bands 2 and 9) happened only in the two entrances that presented exclusively small round seeds (G0061 and G0188). For generalisations, however, it would be necessary to study a larger number of accesses, to relate the possible correlations among those in *P. lunatus*. Castineiras *et al*. (1991), working with 173 accesses of *Phaseolus lunatus* of Cuba, observed a correlation between the characteristic number of colours at the forehead and the forehead primary colour, besides the correlation between form and thickness and length and width of the seed. Just considering the seeds morphologic variables, those authors verified that the quantitative variables presented higher weight in the grouping of the entrances of *P. lunatus*. If those variables should be considered in this work, the accesses will be split in a wider number of groups (Table 2)

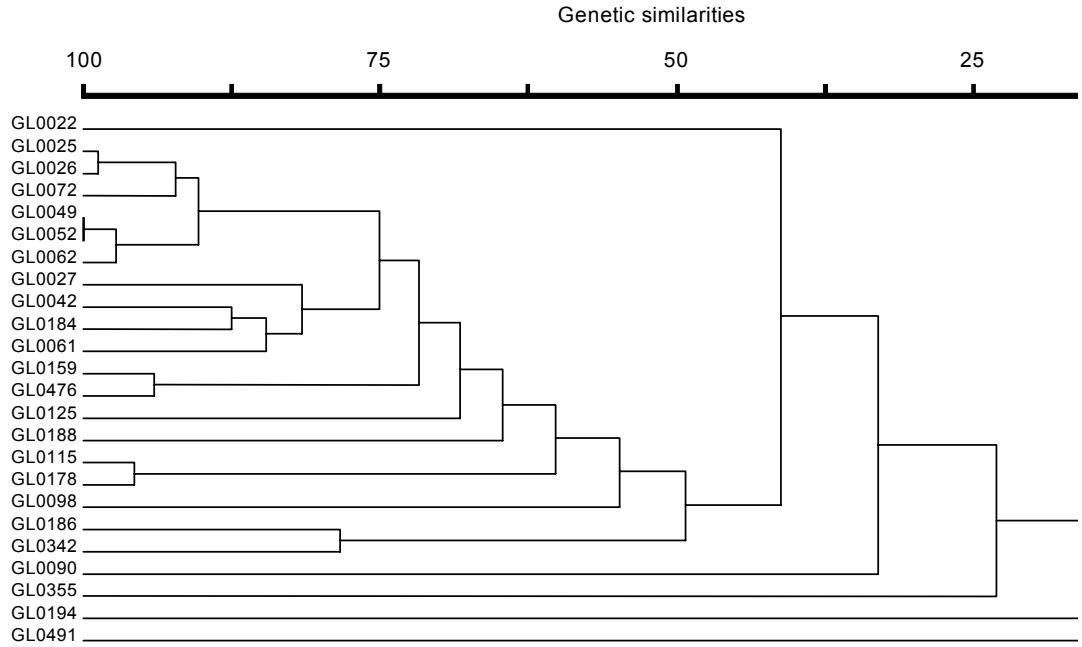

Figure 3. Grouping of *Phaseolus lunatus* accesses using all characters observed: electrophoresis banding pattern and morphological ones

Table 3. Seed electrophorectical pattern, seed type, colour, size, hypocotil and flower colour

Germplasm Code	Electrophoretical pattern	Seed Background colour	Seed Colour Pattern	Seed size	Flower colour	Hypocotil colour
GL0184	I	Black	Speckled	M	Pink	Purple
GL0098	I	Red Dark	Solid	M to B	Pink	Green
GL0178	II	Maroon	Solid	S to M	Pink	Purple
GL0027	III	Brown	Speckled	S to M	White	Green
GL0342	III	Cream White	Solid	S to M	White	Green
GL0042	III	Black	Speckled	S to M	Pink	Purple
GL0194	IV	White	Solid	B	White	Green
GL0491	V	White	Extended Hillum	B	White	Green
GL0061	VI	Brown	Speckled	S	Pink	Purple
GL0188	VI	Red	Solid	S	White	Green
GL0025	VII	Dark maroon	Solid	S to M	White	Green
GL0125	VII	Yellowish rose	Solid	S	Pink	Purple
GL0072	VII	Beige	Speckled	S to M	Pink	Green
GL0159	VII	Cream	Extended Hillum	S to M	White	Green
GL0026	IX	Yellowish maroon	Solid	S to M	Pink	Green
GL0476	VII	White	Mottled	S to M	White	Green
GL0115	VIII	Beige	Solid	S to M	Pink	Purple
GL0186	VIII	White	Solid	M	White	Green
GL0049	IX	Red	Speckled	S to M	White	Green
GL0052	IX	Red	Speckled	M	White	Green
GL0062	IX	Maroon	Striped	S to M	White	Green
GL0090	X	Maroon	Solid	M to B	Pink	Purple
GL0355	XI	Dark maroon	Striped	M	White	Purple
GL0022	XI	Red	Solid	B	White	Green

⋆ S = small (8-12mm length); M = medium (13-17mm); B = big (18-26mm)

The electrophorectical profiles of *P.lunatus* accesses obtained from Cernagen were close to the patterns of total proteins obtained by Lioi *et al*. (1991), with entrances of *P. lunatus* of Cuba. It is observed that, generally speaking, the entrances presented, in agreement with those authors, a pattern more resembling each other than the andean type, mainly for the presence of bands with molecular weights of 26 and 28K Da. On the other hand, most of the accesses here analysed presented the band of 23 KDa, absent of the typical andean pattern. According to Lioi *et al*. (1991), the andean type is frequently associated with big seeded cultivars (lima type). Maciel *et al*. (1999) found a high proportion of the andean type in the state of Rio Grande do Sul.

The results here obtained showed a wide initial variability in *Phaseolus lunatus* accesses collected in different Brazilian areas, demonstrating that, by the morphologic variables, as well as for the electrophorectical pattern, they followed none of the patterns mentioned by Lioi *et al*. (1991): mesoamerican type; sieva mesoamerican; potato seed type; andean type; Big Lima; and mesoamerican intermediary sieva - big limas. Also, there is not a clear correlation among the electrophorectical pattern and coloration and size of the seeds of *P.lunatus*. Similar results were found in other species where electrophorectical variation was not related to morphological characters (Hamrick, 1989; Noh and Minocha, 1990; Nagai *et al*., 1991).

The distance between accesses (Figure 3) was wide enough to support the affirmation that we were working with different species. According to Thorpe (1982), similarity values above 85% were observed for different species in the same genus. However, in this case there were no outgroup species as *P.vulgaris*, *P. coccineus* or *P.acutifolius* that could minimise the variation, being just intragroup variation observed.

It was observed that the SDS-PAGE technique did not discriminate all the accesses here analysed, as in Gomes *et al*. (1984), where every cultivar of *Phaseolus vulgaris* analysed presented its own electrophorectical pattern, which made possible to distinguish one from the others. However, the electrophoresis can be used in complementation to the morphologic characterisation of *P. lunatus* seeds, to separate accesses of this species, aiming to help seed collection and preservation and for breeding purposes.

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