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Research Paper

Genetic Enhancement of Seed Proteins in Pigeonpea – Methodologies, Accomplishments, and Opportunities

Botany

KEY WORDS : Breeding, high-protein, pigeonpea, wild species donor

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ABSTRACT

The food and nutritional security in India is assuming alarming situation as the protein availability in the decade ending 2009 has reduced from the recommended 46 g/head/day to < 25 g. This is leading a wide spread mal-nutrition among children and women. In view of continuing high population growth, this problem will assume even greater concerns in time to come. In the present paper the research work conducted at ICRISAT to breed new high-yielding high-protein lines has been summarized.The newly bred pigeonpea lines have protein between 28-30% and yield as good as cultivars, an estimate on protein yield showed that the cultivation of high protein lines in one hectare will yield an additional 100,000 g protein for the farming families living under subsistence level.

Introduction

Protein is considered a primary building block of every living system because it is responsible for overall growth and development of individuals. There are a number of protein variables in the nature but availability of digestible proteins in sufficient quantities is crucial for growing children of all ages and maintenance of tissues in adults. In general the commonly available sources of proteins are classified on the basis of their origin as vegetable or animal proteins. The problem of protein mal-nutrition among people living under subsistence level is not new, but now it is growing with dangerous proportions, especially in the under-developed and developing countries. This is because the animal proteins are getting dearer day by day and home-grown protein is always threatened by small holdings, low productivity, high cost of inputs, and losses due to various biotic and abiotic stresses; and at the end what is harvested by farmers from their limited resources is not sufficient to meet the protein requirement of individuals or family. Under these situations, the only choice with the farmers is to make best use of their land and resources to create balance between the cash earnings and home-grown cereals and pulses. In this context, pigeonpea (red gram) stands ahead of all the pulses due to its drought tolerance and environment-friendly low cost cultivation. Besides enriching the soil, it produces quality grains with about 22% protein. To meet the future protein needs there is a need to produce more protein per unit area. This is possible by breeding cultivars with good yield and high protein. In comparison to cereals, very little efforts have been made to understand the nature of genetic variation, genetic control, genotype - environment interaction, quality, digestibility of protein and breeding high protein lines. In this review paper an attempt has been made to compile the available information and archive the methodology adopted at ICRISAT in the successful genetic enhancement of seed protein along with some key results.

Proteins in pigeonpea seeds

Being a pulse crop the seeds of pigeonpea are rich in protein and therefore make an ideal combination when eaten with carbohydrate-rich cereals. A generalized sketch of protein distribution in dry seed is given in Table 1. Within a seed the protein is present in all its major portions, and embryo is the as far as the protein concentration is concerned. However its size is very small and it is detached and lost during the process of de-hulling. The cotyledons, the edible portion of pigeonpea seed, contains about 22% protein. The testa has a little protein and it is generally fed to animals after de-hulling as valuable feed. The pigeonpea protein can be separated into four major portions, commonly identified as albumin, globulin, glutelin and prolamin. In the cotyledons globulin accounts for about 60% of the total protein while prolamin is the least among protein fractions. The sulfur containing amino acids (methionine and cysteine) are limited in pigeonpea, but the presence of lysine is significant,

Besides valuable protein the pigeonpea seed also contain certain proportions of some anti-nutritional compounds. The prominent anti-nutritional compounds includes oligo-saccharides such as raffinose, stachyose and verbascose; enzyme inhibitors such as trypsin, chymotrypsin, and amylose; and phenols and tannins. Kamath and Belavady (1980) reported that in pigeonpea seeds there are some unavailable carbohydrates which reduces bioavailability of nutrients. The seed coat has a greater proportion of polyphenols, particularly in the dark seeded genotypes; but once dal is made from the grains these seed coat anti-nutritional factors are of no significance.

Table 1. Generalized information about protein and its important constituents in different parts of pigeonpea seed

Constituents	Whole seed	Cotyledons	Embryo	Testa
Protein (%)	20.5	22.2	49.6	4.9
Protein fractions				
Albumin (%)	10.2	11.4	17.0	2.6
Globulin (%)	59.9	64.5	52.7	26.3
Glutelin (%)	17.4	18.2	21.3	32.8
Prolamin (%)	3.0	3.5	2.7	4.2
Key amino acids				
Lysine (g/100g protein)	6.8	7.1	7.0	3.9
Threonine (g/100g protein)	3.8	4.3	4.7	2.5
Methionine (g/100g protein)	1.0	1.2	1.4	0.7
Cysteine (g/100g protein)	1.2	1.3	1.7	-

Source: Faris and Singh (1990); Singh and Jambunathan (1982)

Variation for protein in primary gene pool

Traditionally, the cultivated pigeonpea [*Cajanus cajan*] germplasm constitute the primary gene pool of genus *Cajanus*. The huge pigeonpea germplasm collection housed at ICRISAT and ICAR harbour a tremendous genetic variation. The first information on the genetic variation for quality traits in pigeonpea was published by Pal (1939) he concluded that as compared to other pulses, pigeonpea has the best combination of nutritive traits with high biological value. Subsequently, Tripathi et al. (1975), Narsimha and Desikachar (1978), Manimekalai et al. (1979) and Singh et al. (1984) also reported a considerable variability for protein content among pigeonpea genotypes. The variation for

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protein content in the germplasm collection reported by Remanandan et al, (1988) needs revalidation because of limited un-replicated samplings from a single location used for characterization. The pigeonpea breeders felt the reliable information on the genetic variation for protein is lacking for launching any programme on its genetic enhancement. Therefore, alternative sources need to be identified for breeding high genotypes in pigeonpea.

Effect of environment on protein content

When any biological system is exposed to external environment, the genotype - environment interaction is inevitable. The extent to which the expression of genotype is influenced by the environment depends on the severity of environmental factor and ability of genotype to resist the changes. In general the quantitative traits are more prone to this interaction as compared to the qualitative traits that are controlled by one or two genes. The literature available in pigeonpea showed significant G x E interaction (Sham, 1976; Singh et al., 1974; Esh et al., 1959; Jain et al., 1986; Singh et al., 1984). In this context, the experiments conducted by ICRISAT and reported by Saxena et al. (1987) showed a large variation for protein content when the same set of genotypes was sown every month at Patancheru and for seven consecutive months at Pantnagar (Table 2). The protein content in cv. Prabhat varied from 22-25 at Patancheru and 25-28 at Pantnagar, respectively. The data showed significant influence of location and months (ANOVA not reported) on the expression of seed protein. Almost similar results were obtained for rest of five cultivars. This variability could be attributed to large variation in temperature and photo-period. These two factors regulated the flowering time in pigeonpea and expose the plants (during reproductive stage, particularity seed development), to different temperatures and this led to variation in the protein content of the seed.

Table 2. Variability in protein content at Patancheru (12 environments) and Pantnagar (7 environments) within a calendar year

Genotype	Patancheru (17° N)			Pantnagar (29° N)		
	Mean	Range	CV%	Mean	Range	CV%
Prabhat	23.4	22-25	5.9	25.9	25-28	4.6
Pusa Ageti	23.8	21-26	6.5	26.7	25-29	6.2
T. 21	24.3	22-26	6.4	26.8	26-29	3.4
No. 148	24.0	21-27	7.0	26.4	25-28	4.5
ST 1	23.6	22-25	3.2	26.5	25-28	3.7
PDM 1	23.6	20-27	8.6	26.4	25-28	3.8

Source: Saxena et al., (2002)

Protein alterations during de-hulling and storage

De-hulling: Pigeonpea is predominantly consumed as *dal* (decorticated dry splits) that is prepared both domestic and commercial levels by removing the seed testa and separating the two cotyledons. In this process the *dal* recovery is around 60 -70%. This means a considerable proportion of seed is lost before *dal* is consumed. According to Reddy et al. (1979) relatively more protein is accumulated in germ (embryo) and outer layers of cotyledons. During the process of de-hulling both germ as well some outer portion of the layer of cotyledon is lost and that means loss of protein that is available for human consumption. This is a real constraint with pigeonpea and even with the most advanced milling technology such losses cannot be stopped completely (Kurien, 1981).

Storage: Most farming families store pigeonpea grains for round-the-year consumption and its small quantity is de-hulled and consumed as per the domestic necessity. Storing of seeds for a longer period result in the loss of seed quality, besides physical damage by storage pests. Daniel et al. (1977) reported that lysine, threonine, and protein efficiency ratios were adversely affected when the seeds were stored in jute bags. Uma Reddy and Pushpamma (1981) also observed reduction in amino acid contents in the infested seed samples and decline in lysine content was greater than those of methionine and tryptophan.

Inheritance of protein content

Information on the genetic nature of a trait is essential for effective breeding results. This type of information for seed proteins is very limited in pigeonpea. This may be due to lack of good genetic (diverse) materials, lack of laboratory facilities or low research priority. Dahiya and Brar (1977) and Durga 1989) reported a strong maternal influence on the expression of protein content of an F_1 individual. Dahiya (et al. (1977) also reported that in pigeonpea at least 3-4 genes controlled its protein content. Reddy et al. (1979) observed that the magnitude of heterosis for protein was in the negative direction. Durga (1989) also reported that protein content was under additive and complementary gene effects and low protein was dominant or partially dominant over high protein. Saxena and Sharma (1990) while reviewing the subject concluded that in pigeonpea both additive and non-additive genetic variations were important for the expression of seed protein.

Breeding high-protein pigeonpea

Selection of parents

To achieve success in any breeding programme it is important to select right parents along with correct method for handling breeding populations. For selecting parents to breed high protein high yielding lines the breeders at ICRISAT made a choice to use wild relatives of pigeonpea. The reason for selecting the wild species from secondary gene pool was their easy crossability with cultivated type and the quantum of protein in the wild species was much greater than cultivated type (Table 3).

C. scarabaeoides: It is source of A_2 cytoplasm for male sterility that has been exploited commercially. Viney plant with thick, spongy small trifoliate ovate leaves, heavy bearing with hairy pods, 3-5 dark brown to grayish black round seeds of smaller size (2.3 g) in each pod, high protein content (28.4 %) resistant to drought, Helicoverpa and Sterility Mosaic Disease. This male-sterile source was used in developing experimental hybrids in Gujarat state of India (Fig 1).

C. sericeous: Erect shrub, to 1.5 m, branched,grey green, leaflets palmately arranged, white-hairy, oblanceolate, quite narrow. Flowers 1-3 in leaf axils, pale yellow, pods 1.1 to 1.3 cm, with two to three (mostly two), grey to black seeds with high (29.4 %) protein content (Fig 2).

C. albicans: It is secondary gene pool, distributed in India and SriLanka. A climber with obovate to rounded leaflets, grey-hairy below, flowers yellow, sometimes flag brown at the base, quite fertile when fruiting, pods 1.5-3.5 cm with short adpressed hairs, sutures sturdt, 5-7 grey seeds with black mosaic having small seed size (12-16 mg). It is viney plant type with small leaves and poor pod setting (***Fig 3).***

Table 3. Three wild relatives of pigeonpea used as protein donors

Wild species	Protein %	100-seed Wt. (g)	Seed Colour	Plant type
C. scarabaeoides	28.4	2.3	Dark	Trailing
C. sericeous	29.4	1.9	dark	Erect
C. albicans	30.5	2.8	dark	Creeper
BDN 1 (C)	22-1	9.8	Brown	Erect

Source: ICRISAT

Breeding methodology

Before hybridization single plants of each species were examined for seed protein content in the laboratory and only those conforming high protein were used in hybridization. The crosses were made late in the season (February) on the selected plants using them as female parents. The crossing success was low (<5%). In the next cropping season the F_1 generation was raised in a glasshouse and multiple harvesting of mature pods was done to get more seeds for raising a large F_2 population of each cross.

The crucial phase of pedigree selection started in F_2 generation. To avoid natural out-crossing of the selections, all the populations in each generation were grown under insect-proof nets and the selection scheme was carefully designed. In F_2 generation about 3000 seeds were sown and only 70-80% germinated due to hard seed coat problem. The crop was raised under irrigation. As expected the populations segregated for plant type, size, shape, and colour of pods and seeds, and various other morphological traits.

The top most selection criterion was protein content. For this, each F_2 plant was numbered and at maturity seeds were harvested and soon sent to laboratory for protein analysis on duplicate *dal* samples using Auto-technicon method. Each population was divided on the basis of protein content into high protein (>25%) and low (control value + 2 protein units). protein group. In F_3 single plant progenies of all the high protein selections were raised @ 100 plants/progeny. Within each progeny a mild selection for plant type was exercised and the individuals carrying wild species traits such as creeping and abnormal growth patterns were rouged and within each progeny 10 plants were selected randomly and their protein determinations were made. In this generation wider segregation for protein was observed and the plants with high (>25%) protein content were selected. Among the selections the plants small seeds (< 6 g/ 100 seeds) were discarded and the rest were selected for progeny row evaluation. In the next three generations (F_3, F_4 and F_5) the same exercise was continued; and in each generation selection for seed type and plant type was continued with greater emphasis on protein content. In F_5 generation some segregants with 28% protein were obtained and this was an encouraging sign. In the next four generations (F_6 to F_9) a few selections with protein ranging from 28-32% were also obtained. During this period selection for seed type was also exercised and seeds of about 10g /100 seeds were selected. In F_{10} generation the first set of yield trials of high protein ($\geq$ 28 %) lines was conducted. A mild selection for seed type/colour and protein continued to purify the lines along with their agronomic evaluation. The results from evaluation (Table 4) were very encouraging and provide an opportunity to breed high-yielding high-protein pigeonpea cultivars.

Agronomic evaluation of high protein lines

In Table 4 data related to two yield trials of high-protein lines are summarized. In the evaluation of non-determinate lines, the yield of the top two test lines (HPL 40-5 and HPL 40-17) was over two tonnes/ha and it was similar to that of the control BDN 1 (2.02 tonnes /ha). These lines also compared well with control in maturity as well as seed size. The protein content of the high protein lines, however was significantly higher than the control (23.2%). The advantage of the high protein lines was reflected in the total protein harvest from unit land. The similar results were recorded from the evaluation of determinate high protein lines (Table 4).These results demonstrated that in pigeonpea seed yield, seed size and protein can be enhanced simultaneously.

Table 4. Seed yield and protein harvest from high protein F_{10} lines at Patancheru

Genotype	Maturity (days)	100-seed wt (g)	Yield (t/ha)	Protein (%)	Protein yield (kg/ha)
HPL 40-5	169	9.6	2.10	26.9	452
HPL 40- 17	169	8.5	2.07	26.5	440
BDN 1 (c)	168	9.6	2.02	23.2	373
(SE +-)	+0.9	0.18	0.16	0.46	37.3
CV (%)	0.9	3.4	17.3	3.0	17.0
HPL 8-10	163	10.5	1.66	26.5	353
HPL 8-16	162	10.5	1.57	27.4	344
I C P L 211(C)	162	14.3	1.46	21.6	251
SE (+)	1.1	0.15	0.19	0.21	38.5
CV (%)	13	2.5	27.0	1.7	25.8

Source: Singh et al. (1990)

Stability of high protein content

Studies conducted to understand the effect of diverse environmental factors on the protein content of high protein lines yielded very useful results. The evaluation of the lines across wide locations in six states of south, north and central India showed that although minor (2-3 protein units) differences were observed, but the difference between the high and low protein cultivars were maintained (Table 5). HPL 24 appeared to be the best with >30% protein recorded at each place. Similarly, evaluation of high-protein lines over six years showed that the protein content of each high protein selection was higher in each year the values were much higher than the control (Fig 4). These observations indicated that high protein trait derived from the wild relatives of pigeonpea were very good and will not pose any difficulty in breeding high yielding hgh protein lines for cultivation.

Table 5. Stability of protein content among four high-protein selections

Location	HPL 24	HPL 25	HPL 26	HPL 28	CONTROL	SE ±
Patancheru (AP)	31.3	28.6	29.7	27.8	23.3	0.26
Jalna (Mah)	32.2	28.9	29.7	30.4	23.1	0.69
SK Nagar (Guj)	30.9	28.4	29.0	27.3	21.4	0.36
Gulbarga (Kar)	32.1	29.9	-	27.6	23.0	0.49
Gwalior (MP)	32.3	30.4	28.2	27.3	22.0	0.71
Hisar (Har)	31.1	29.6	31.7	29.2	24.5	0.51
Mean						

Saxena et al. (2002)

Biological evaluation of high protein lines

The biological evaluation of the protein-rich genotypes is the ultimate test of the efforts made in breeding these lines. This test will determine if the additional protein can be utilized in growth and development of the individuals. In the present case this information becomes more important because the high protein trait was transferred from wild species. The test lines were significantly superior to the control in their protein content (Table 6). The differences in the major protein fractions of the high and normal protein lines were large.in comparison to controls (60.3 to 60.5), the globulin fraction was higher (63.5 to 66.2).this variation was not large enough to influence the amino acid profile of the high protein lines (Singh et al., 1990).

The biological evaluation of the test lines (Table 6) showed that the high-protein lines were significantly superior to in utilizable protein (Singh et al., 1990). It was also reported that the high-protein lines were nutritionally superior to normal cultivars because of their greater sulfur-containing amino acids. They also concluded that whole seeds of high protein lines for animals and

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dal for human beings is nutritionally beneficial; and such lines can help, if promoted appropriately, in addressing the issues related to rural nutrition.

Table.6. Comparison of high protein pigeonpea line and control cultivar for protein and its constituents and biological parameters

Item	High protein line HPL 8	High protein line HPL40	Control line (ICPL 211)	SE
Constituents				
Starch (%)	54.3	55.6	59.3	±0.30
Protein (%)	28.7	31.1	23.1	±0.09
Albumin (%)	9.1	8.0	8.6	±0.34
Globulin (%)	63.5	66.2	60.3	±1.08
Glutelin (%)	20.2	19.7	22.8	±0.75
Prolamin (%)	2.9	3.2	2.1	±0.06
Cysteine	0.8	0.8.	0.7	±0.01
Biological parameters				
Total protein digestibility	83.7	82.9	85.7	±2.14
Biological value	67.0	65.3	62.9	±1.68
Net protein utilization	56.1	54.1	53.9	±1.06
Utilization protein	15.5	16.7	12.3	±0.25

Source: Singh et al. (1990)

Discussion

The world-wide commercial exploitation of dwarfing genes in rice and wheat to breed fertilizer responsive high yielding varieties saved the world from hunger. But this was not enough for the nutritional security of masses which besides calories also needed protein and other vital nutrients and the problem of global nutritional security is still a major issue before the policy makers. According to UNICEF (2008) "Food and nutritional security is said to be achieved when adequate food (quality, quantity, safety, socio-economic acceptability) is available and accessible for and satisfactorily used and utilized by all individuals at all time to live a healthy and active life". Considering this wisdom, most developing and under-developed countries need to achieve a lot on the food front, and India is no different where its population is growing with an alarming rate. According to World Bank (2012) statistics by 2020 India will have 1.39 billion people to feed and 35 % of its population (445 million) are poor with daily income of US $ 1.25. They further estimated that about 50% pregnant women are anemic. Also about 74% of the children are anemic and 43% underweight. According to Reddy (2013) "malnutrition is not the result of a single cause but is multi-faceted problem with other complex factors like poverty, health care, ignorance and policies". In a recent publication by Shalendra et al. (2013) reported that in the decade starting 2000, the consumption of cereals, pulses and sugar has come down both in rural and urban India by 10-11%. Of these commodities the reduction in consumption of pulses is a matter of concern and against the recommended intake of 42 g protein /day/head in the rural areas, it has come down in 2009 to 23 g and 27 g in urban areas. So, overall situation about food and nutritional safety in India is grim and a lot needs to be done to promote both cereals as well as protein-rich pulses.

In the context of the importance of pulses in rural diet (Bidinger and Nag 1981) and concern that of late the consumption of pulses in both rural and urban areas in decreasing (Shalendra, et al, 2013) it becomes more important to review the policies related to production of pulses be reconsidered and new crop cultivars which perform well under low input subsistence agriculture. Under these testing situations no crop other than pigeonpea stands out. The present attempt to breed high yielding high protein lines is an option before the breeders to address the issues related to mal-nutrition. The genotypes like HPL 40 and HPL 8 are capable of yielding about 100 kg/ha extra protein; and @ 46 g of protein requirement per day, the high protein lines can theoretically support another 2000 heads, as far as protein requirement is concerned. A good beginning has now been made in this direction and hope the concerned persons will reap the advantage of this breakthrough.

Conclusions

From this extended exercise the following conclusions are drawn:

- Protein mal-nutrition in both rural and urban areas is on rise and this issue needs to be addressed at national level,
- Protein content in the new breeding materials is high and can be used to breed high-yielding high-protein cultivars.
- High protein content is controlled by recessive oligo-genes.
- It is not possible to select high protein lines in early generations
- Response to selection for enhanced protein in later generations was productive.
- There is no yield penalty while breeding for high-protein.
- Development of high-yielding high-protein lines is possible.
- There is no adverse linkage between seed size and protein content.
- The biological value of high-protein lines is high.
- There is no strong anti-nutritional compound in the high-protein lines.

Fig 1. *Cajanus scarabaeoides,* **a wild relative and donor of high protein**
(Source: ICRISAT)

Fig 2 *Cajanus sericeus,* **a wild relative and donor of high protein Source: ICRISAT**

Fig 3 *Cajanus albicans,* **a wild relative and donor of high protein Source: ICRISAT**

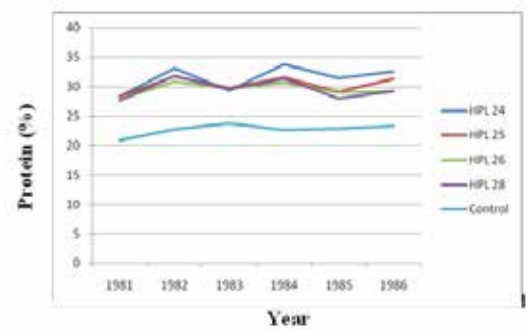

Fig 4. Stability over years of protein content among four high-protein selections

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