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REVIEW ARTICLE

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Breeding high-protein pigeonpea genotypes and their agronomic and biological assessments

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Abstract

Proteins, inevitable for nutritional security of human beings and legumes, by far, are the cheapest source of this vital nutrient. The escalating prices and never halting population growth limit the per capita availability of protein-rich legumes. In view of limited land resource and need to grow other food crops, the greater protein harvests are possible only by increasing the protein levels of popularly grown legumes. In this context, attempts were made for raising the protein content in pigeonpea [*Cajanus cajan* (L.) Millsp.] through traditional plant breeding tools. For this, the high-protein trait was successfully transferred from wild relatives of pigeonpea to the cultivated types. In the derived inbred lines, the protein content was significantly enhanced from 20% - 22% to 28% - 30%. Two high-protein lines HPL 40 and HPL 8 also produced 2100 and 1660 kg/ha grain yield, respectively. This simply means that, in comparison with traditional cultivars, the cultivation of high-protein lines will provide additional 100 kg/ha of digestible protein to the farming family. This paper, besides describing the breeding procedures, also discusses the accomplishments of this breeding endeavour with respect to its various nutritional and biological properties.

KEYWORDS
Cajanus cajan, high-protein selections, nutritional parameters, wild species donors

1 | INTRODUCTION

The poverty-driven protein-energy malnutrition is a key nutritional security issue, particularly in south Asia and Africa—the most populace regions of the world. The FAO (2019) statistics show that over 10.8% of the global population is undernourished with about 149 m children suffering owing to undergrowth and about half of them fail to survive due to various health issues related to undernutrition. The famous 'Green Revolution' of the 1970s, led by dwarf rice and wheat varieties, provided the much-needed calorie-filled food cover and saved the world from widespread hunger. But in the process, the R&D of pulses was put on the back burners. Since the animal proteins are getting dearer with time, the use of home-grown pulses remains the primary protein provider. Because these pulses are deficient in amino acids like methionine and cystine, Hulse (1977) recommended that a mixture of 70% cereals and 30% legumes will make a good balanced diet. According to Kurien et al. (1971) and Daniel et al. (1970), the enhancement of pulse supplement in cereal-based diets markedly improved the nutritional quality of diets. Also, a cereal:pulse ratio of 3:1 for young children, 5:1 for women and 6:1 for men was considered ideal from nutrition point of view. Unfortunately, for most Indian villagers such food standards are too luxurious to afford on a sustainable basis. A survey of Indian villages carried out by Bidinger and Nag (1981) revealed that most regular rural diets provide about 10% of the protein, 5% of energy and 21.7% of the required lysine and this reflects their below par nutritional levels.

The availability of home-grown plant-based protein is often restricted by the limitations of farming land, low yields and expensive inputs. Pigeonpea [*Cajanus cajan* (L.) Mills.] with 20% to 22% protein and presently grown on over 5 m ha (FAO, 2021), appears to be the most ideal crop from the points of view of adaptation and production (Saxena et al., 2021). With the productivity plateauing at around

700 kg/ha, the present-day pigeonpea cultivars cannot provide enough protein to ease out the issue of malnutrition. Therefore, to enhance the contribution of pigeonpea-based protein in tackling the malnutrition issue, increasing the protein harvests from the available land resource seems a logical approach. This would be possible if some high-protein cultivars were developed without losing their productivity. To achieve this goal, the pigeonpea researchers launched a project to transfer the high-protein trait from wild species into the cultivated types. This paper besides summarizing different breeding and laboratory procedures used in the study also highlights the key accomplishments.

2 | VARIATION FOR PROTEIN CONTENT IN PIGEONPEA GERMPLASM

Germplasm resource of a crop is known to serve as a reservoir of diverse genetic materials where breeders exercise gene mining as per their needs. Pigeonpea gene banks at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) and other research institutions house approximately 15,000 accessions (Upadhyaya et al., 2016). This collection harbours vast genetic variation for different seed and plant traits.

Pal (1939) published the first research on certain quality traits of pigeonpea and reported that in comparison with other pulses, pigeonpea has the best combination of different nutritional traits with high biological value. Esh et al. (1959) reported a considerable variation for protein content among pigeonpea genotypes. On the contrary, Swaminathan (1973) found a little variation for protein in 2000 genotypes. The researchers including Tripathi et al. (1975), Hulse (1977), Narsimha and Desikachar (1978) and Manimekalai et al. (1979) reported a considerable variation for protein content among pigeonpea genotypes. Srivastava and Vasishtha (2012) reported 20.13% to 23.35% protein in different pigeonpea cultivars. In the routine germplasm characterization process at ICRISAT Remanandan et al. (1988) reported 22% to 28% protein in the primary gene pool. However, the odd high-protein values reported by them in some accessions could not be reconfirmed for their use in the protein breeding programme (U. Singh, pers. com.)

The inferences drawn from these reports were that the variation for protein content in the primary pigeonpea gene pool is limited and these cannot be used as donors in any high-protein breeding programme. This shifted the attention towards secondary gene pool to identify a crossable wild species for use as high-protein donor. The protein analysis of random samples drawn from the crossable wild species revealed that, in contrast to the domesticated types, these wild relatives of pigeonpea have distinctly greater protein contents. Therefore, from this resource, three high-protein wild species—*Cajanus scarabaeoides*, *Cajanus albicans* and *Cajanus sericeus*—were selected as donor parents.

3 | PIGEONPEA PROTEINS AND THEIR SEATS WITHIN THE SEED

Following the successful fertilization of pigeonpea flowers, the pod shell starts growing rapidly and the full pod length is attained in about 3 weeks. During this period, the ovules inside the pod remain intact but do not gain weight. In the following fortnight, the ovules grow rapidly to reach their optimum size. Meiners et al. (1976) observed that in legumes, the contents of various minerals and trace elements such as calcium, iron, zinc, magnesium and copper remained the same throughout the period of ovule development. The crude fibre content in the growing pigeonpea seeds increased slowly with maturation. The proportions of protein in the growing seeds declined gradually, but their starch accumulation gained the pace (Singh et al., 1991).

A mature pigeonpea seed is made up of about 85% cotyledons, 14% seed coat and 1% embryo (Faris & Singh, 1990), and the protein molecules are distributed in all the major portions (Table 1). The seed coat has large (about 30% to 35%) proportion of fibre and negligible amount of protein. Each pigeonpea seed has a pair of edible cotyledons, joined together with natural gums. These are rich in both carbohydrates (65% to 70%) and proteins (18% to 22%). The embryo of pigeonpea seed is very small in size but predominantly (about 50%) made up of proteins.

Pigeonpea proteins have four major portions, commonly identified as albumin, globulin, glutelin and prolamin. Of the total cotyledonous proteins, about 60% is globulin while prolamin content is the least. The sulphur-containing amino acids (methionine and cysteine) are present in cotyledons and embryo, but their proportion is only about 1%. On the other hand, the proportion of lysine is significant (Singh & Jambunathan, 1982). According to Singh and Eggum (1984), the content of sulphur-containing amino acids in pigeonpea is not linked to its low methionine. In comparison with other protein fractions, globulin is rather inferior in sulphur-containing amino acids while albumin has greater amino acid content. Besides valuable

TABLE 1 Generalized information about the distribution of protein and its key constituents in different parts of a pigeonpea seed.

Constituent	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 (g/100 g protein)				
Lysine	6.8	7.1	7.0	3.9
Threonine	3.8	4.3	4.7	2.5
Methionine	1.0	1.2	1.4	0.7
Cysteine	1.2	1.3	1.7	-

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

protein, pigeonpea seeds also contain certain proportions of some antinutritional compounds and these include oligosaccharides (raffinose, stachyose and verbascose), enzyme inhibitors (trypsin, chymotrypsin and amylose) and phenols and tannins.

4 | GENETIC CONTROL OF PROTEIN CONTENT

Information on the genetic control of a trait is helpful in breeding, particularly in formulating hybridization and selection schemes. In case of pigeonpea, such information with respect to protein is inadequate; and this could be due to low research priority or limitation of resources. Casey and Domoney (1984) reported that most of the storage protein genes exhibit simple codominant Mendelian inheritance. Williams (1948) and Qureshi et al. (2013) concluded that in major legume crops the protein content is controlled by dominance, partially dominance, additive and/or nonadditive gene actions. McKendry et al. (1986) reported partial dominance of low protein with additive effects in soybean (*Glycine max*). Gaur et al. (2016) and Vijaylakshami et al. (2001) observed a continuous variation for protein content in crosses involving high- and low-protein lines of chickpea (*Cicer arietinum*). In these crosses, the protein was also found to be linked to seed size and flower colour.

In pigeonpea, Dahiya and Brar (1977) and Durga (1989) reported significant maternal effects for protein content in F_1 generation. Similar observations were also reported in soybean (Singh, 1969) and beans (Leleji et al., 1972). Dahiya et al. (1977) reported the presence of three to four protein-controlling genes in pigeonpea. Reddy et al. (1979) observed that the magnitude of heterosis for protein was in the negative direction, suggesting the recessive nature of the genes. Durga (1989) reported that the protein content in pigeonpea was under additive and complementary gene action and low protein was dominant or partially dominant over high protein. In contrast, Dahiya et al. (1977) reported that additive genetic variances were not important in controlling seed proteins in pigeonpea. They further concluded that pedigree breeding for protein may not be very effective due to low heritability and possible environmental influences.

In *Cajanus* interspecific hybrids, the high-protein trait was found to be controlled by dominant genes with their F_2s exhibiting quantitative variation (Reddy & Singh, 1981). In the interspecific soybean crosses, Weber (1950) reported the presence of three genes with partial dominance which controlled the high-protein content. On the contrary in the interspecific oat cross, Campbell (1970) reported the presence of duplicate epistatic gene system that controlled low-protein content.

5 | MATERIALS AND METHODOLOGIES

To breed high-protein cultivars, a search was made for useful donors with distinctly high-protein contents. Because the primary *Cajanus* gene pool lacks such resource, the high-protein wild species, which could be crossed easily with the domesticated types, were selected. Such interspecific breeding programmes, however, often encounter difficulties at one or more stages from hybridization to selection.

5.1 | Selection of parental materials

5.1.1 | Donor species

As mentioned earlier, for the genetic enhancement of seed protein in pigeonpea, three crossable wild species representing secondary gene pool were selected. These were *C. scarabaeoides*, *C. sericeus* and *C. albicans* (Table 2). The key information about these species, as highlighted here, was described by van der Maesen (1986).

i. *C. scarabaeoides* (L.) van der Maesen comb. nov. (=*Atylosia scarabaeoides* L.) is a creeper-climber (Figure 1) with winding pubescent branches. It is widely distributed in parts of Asia, Australia and Africa. It is generally found growing in open grassland, dry scrub vegetation and deciduous monsoon forests. It has trifoliate ovate leaves with small glandular leaflets. Flowering in this species is profuse and continues for a long time. Racemes are short with one to six yellow flowers in each. Most flowers drop before fertilization but still produces a lot of small shattering type pods. The pods are oblong and 1–2 cm long, and on average, each pod produces three to five dark brown/grey seeds of small size

TABLE 2 The high-protein wild species donors and domesticated pigeonpea lines used in breeding high-protein and high-yielding lines.

Parental line	Protein (%)	100-seed wt. (g)	Seed colour	Plant type
Wild species donors				
Cajanus scarabaeoides	28.4	2.3	Dark	Trailing
Cajanus albicans	30.5	2.8	Dark	Creeper
Cajanus sericeus	29.4	1.9	Dark	Erect
Recipient cultivars				
Pant A3	22.7	7.5	Brown	Erect
T21	24.4	7.5	Brown	Erect
Baigani	23.7	11.2	White	Erect

FIGURE 1 Figure represents phenotypic characteristics of three wild relative species, namely, *Cajanus scarabaeoides* (L.) van der Maesen comb. nov. (=*Atylosia scarabaeoides* L.), *Cajanus sericeus* (Benth.) van der Maesen comb. nov. (=*Atylosia sericea* Benth.) and *Cajanus albicans* (W. & A.) van der Maesen comb. nov. (= *Atylosia albicans* Benth.).

(2.3 g/100 seeds). Seeds of this wild species are rich in protein content (28.4%).

ii. *C. sericeus* (Benth.) van der Maesen comb. nov. (=*Atylosia sericea* Benth.) is mainly found in the ghats of western and eastern India. It is a densely branched erect shrub (Figure 1), about 1 m tall. The branches are erect and striate. The leaves are trifoliate and glandular with small greyish green leaflets. Its racemes are sessile, axillary, one to three yellow flowers are borne in leaf axils. The oblong pods are small (11–13 mm long), and on average, they contain two rectangular-round grey and black seeds with cream mosaic. Seeds of *C. sericeus* are rich in protein (29.4%).

iii. *C. albicans* (W. & A.) van der Maesen comb. nov. (=*Atylosia albicans* Benth.) is a perennial climber with woody base (Figure 1). It is distributed in peninsular India and Sri Lanka in tropical dry deciduous forests. Its branches are long, green with whitish pubescent, and leaves are trifoliate with obovate to rounded leaflets. Racemes are lax with one to four small yellow flowers. The pods are oblong, 1.5–3.5 cm long and covered with short dense hairs. Seeds are rectangular round with grey and black mosaic colour. On average, each pod contains five to seven dark grey seeds. The seeds are small but have about 30% protein.

5.1.2 | Recipient cultivars

Three early maturing pigeonpea cultivars with good agronomic base were selected for interspecific hybridizations. Among these, Pant A3 is determinate, while Baigani and T21 are non-determinate in growth habit. These cultivars are early maturing and known for their wide adaptation, high yield and commercially accepted seed traits (Table 2).

5.2 | Development of breeding materials

As compared with domesticated genotypes, the interspecific hybridizations are always difficult. Pundir and Singh (1985), Reddy (1990) and Dundas (1990) recorded that success in the interspecific hybridizations varied considerably from <5% to 35%. In the present study, three pigeonpea cultivars 'Baigani', 'Pant A3' and 'T 21' were crossed as female parents with wild species to produce six F_1 hybrids. The emasculations and pollinations were done between 1000–1600 h. Only two young buds per florescence bunch were used for hybridizations.

To develop interspecific populations, 30 plants of each parental line were sown in a crossing nursery. Before commencing hybridizations, each plant of each wild species was subjected to protein determination, and only those confirming the high-protein trait were used in crossing in the ratooned (regenerated) plant growth.

5.3 | The growing environment

Most biological plant systems are vulnerable to environmental changes, but their intensity may vary from one environment to the other. Besides this, the host plants may also play an important interactive role in the expression of a phenotype. The key factors in this act are the severity of specific environmental factor(s) and inability of plants to resist the changes. In a multienvironment experiment, Saxena and Sawargaonkar (2015) recorded a large variation for protein content when a set of five pigeonpea genotypes was sown in different months at more than one location. This variability was attributed to large variation in the prevailing temperatures and photoperiods. These two factors regulate the flowering time in pigeonpea and thereby they exposed the plants to different environments during reproductive stage which led to variation in the protein content of seed. To minimize such variation, each year, the breeding materials were sown in the first week of July with similar agronomic package. The insect-aided cross-pollination (Saxena et al., 2016) is another hindrance in pure line breeding in pigeonpea. If not controlled, the resultant out-crossed (natural hybrid) plants will not breed true and adversely affect the heritability and genetic advance. Therefore, to exclude the pollinating insects from the breeding block and to maintain genetic purity, all the breeding materials were grown under insect-proof nylon-net cages, fixed on aluminium frames.

5.4 | Field plot techniques

Most wild *Cajanus* species possess hard seed coat which protects their seeds from dangerous insects, water-logging and various soil-borne pathogens. The hard seed coat generally delays germination by 2–3 weeks or even more. The adverse effects of late germination on plants are visualized in the form of stunted seedlings, inadequate canopy development and low productivity. To overcome this bottleneck and speed up the germination, each seed was scarified with a sharp blade. Because the hard seed coat in the wild species is controlled by a single dominant gene (Reddy, **1990**), all the seeds were also scarified for raising F_1 and F_2 generations.

For planting breeding materials in each season, a basal doze of diammonium phosphate was applied at 100 kg/ha. For good drainage, ridges, 75 cm apart, were constructed in field along the slope. The seeds were manually placed at 2–3 cm depth with interhills distance of 30 cm. In progeny row evaluations, a popular control cultivar BDN 1 was sown after every five test plots. The experimental area was irrigated for uniform germination, and further irrigations were given as and when required. From flowering to maturity, the crop was monitored by plant protection team and sprayed with chemical insecticides as and when found necessary.

5.5 | Protein determinations

To minimize the protein-determination errors, most of the operations were carried out mechanically. After harvesting and cleaning, the seeds were oven-dried (at 55°C for 24 h), and an electronic seed counter was used to take random samples of 100 seeds. The protein estimations were done on the decorticated seed samples, and their testa layers were removed using a tangential abrasive dehulling device; and grinding of the decorticated grains/splits was done using Udy Cyclone Mill. The nitrogen estimations of the samples were done using Technicon AutoAnalyzer, and the protein estimations were done by multiplying the nitrogen readings by a factor 6.25. For each genotype, two samples were taken, and their mean values were used for selection of individual plants (for details see Singh et al., 1990).

5.6 | Biological assessment of high-protein selections

Singh et al. (**1990**) conducted this exercise using feeding trials involving metabolic cages and Wistar male rats. The estimates of true protein digestibility, net utilizable protein and overall biological efficiency of high-protein genotypes were determined (Singh et al., **1990**).

6 | RESULTS AND DISCUSSION

For genetic improvement of protein in some food crops, the breeding methods such as mutations, pedigree selections and backcrosses were tried with some quantifiable genetic gains. The present breeding endeavour, the first in pigeonpea, was quite complex because it involved simultaneous improvement of protein and some key traits such as yield and seed size, particularly in the backdrop of strong linkage drag in the interspecific populations and natural cross-pollination.

6.1 | Development of breeding populations

Deodikar and Thakar (**1956**) were the first to create successful interspecific (then intergeneric) hybrids by crossing *Cajanus cajan* with *Cajanus lineatus* and *C. sericeus* to establish the genetic affinity among these species through cytological evidences.

In the present case, most of the pollinated buds dropped and only 3% to 11% crossing success was obtained in different crosses. Poor seed set recorded in these crosses may be attributed to various cytological or physiological reasons leading to poor pollen germination, restricted pollen tube growth, ineffective fertilization or ovule abortion (Dundas, **1990**; Pundir & Singh, **1985**; Reddy, **1990**). In F_1 generation, each hybrid plant was examined for its leaf morphology marker; and those seedlings matching with their respective female parent were considered self-pollinated and discarded. All the F_1 hybrid plants were given extra care to allow multiple harvests for raising large F_2 populations. Some of the hybrid plants also exhibited partial male sterility and produced only a few pods; and this may arise due to genetic divergence of the parents which often leads to various premeiotic or postmeiotic abnormalities (Dundas, **1990**).

The crucial phase of breeding for high protein started at F_2 stage. In each cross, 800–1000 seeds were sown under insect-proof nets, but only 70% to 80% germinated due to problems such as hard seed coat, formation of soil crust or seedling blight disease. As expected, the F_2 populations segregated for different traits but the emergence of abnormal seedlings (multiple shoots, twisted shoots, albino seedlings and deformed and multifoliate leaves) was interesting. In this generation, many plants showed the traits of wild species; and these were due to the strong linkage between the traits of wild species. Such associations are also known to alter the normal segregation and independent assortment of genes. These linkages may be weak or strong. The weak linkages are no threat in breeding as enhanced recombination, and large population can overcome this situation. In contrast, the strong linkages adversely affect the selection programme. Under such situations, referred to as 'linkage drag', the linked traits inherit together (with various degrees) and limit the production of recombinants. This makes the transfer of the target gene(s) from wild species difficult.

At maturity all the plants were harvested and assigned identification numbers. In the next season, F_3 single-plant progeny were raised. In this generation, seed germination improved, and the frequency of deformed seedlings also reduced significantly. In each F_3 progeny, all the plants were harvested separately, and those with wild species-type seeds were discarded. In F_4, many single-plant progeny were grown, and the plants with abnormal growth traits such as inhibited growth, twin seedlings, twining branches, flat main stem, modified

leaves, altered floral and pod morphology were discarded. Besides these, at maturity, each plant in field was examined for its seed traits, and those matching with their respective wild species parent were discarded. The rest of the plants were harvested individually.

6.2 | Selections for protein content and agronomic traits

The selection for protein was delayed, and the first round of protein determination in the project was carried on F_5 seeds. For this exercise, 100 seeds from each plant were sent to quality laboratory for protein analyses. Within in each F_5 progeny, all the plants were assessed for their protein content. The F_5 data of different crosses showed a wide range for protein content (Table 3); and in some segregants, the protein content almost touched the donor species mark. Because our primary objective was to transfer the high-protein trait in to cultivated types, the segregants with protein content approaching the respective pigeonpea parents were discarded, and the rest were advanced to F_6. From each progeny, 5–10 top ranking segregants were selected for generation advance, and the rest were preserved. In F_6 generation also, the same procedure was followed. The F_7 selections were grown in progeny rows, and every plant in each progeny was subjected to protein analysis. Because these progeny achieved notable uniformity for various plant and grain characteristics, their progeny-based data were also collected on different ancillary traits. Subsequently, the selection for seed size, shape and colour was also performed. The progeny with 100-seed weight of ≤6 g and abnormal seed shape or colour were rejected. The selected progeny were in both determinate and non-determinate growth habits and acceptable seed size, shape and colour. These were evaluated in preliminary station trails. The self-pollinated seeds of these lines were maintained for future testing programmes.

To make the selection process clearer, two examples from each of the three crosses are given in Table 3. In cross Baigani × *C. scarabaeoides*, the F_4 selection number 515 was outstanding throughout with protein per cent of 28.5% in F_5, 29.3% in F_6 and 29.5% in F_7 generation. The F_7 progeny performance demonstrated that in this genotype, we successfully recovered the protein content of its wild species donor *C. scarabaeoides* (28.4) and seed size of pigeonpea cultivar Baigani (11.2 g/100 seeds).

6.3 | Correlations between seed size and protein

Optimum seed size (10–12 g/100 seeds) in pigeonpea is necessary to meet its commercial milling requirements. During breeding for high protein, a range of seed size was found among the high-protein selection. To develop a selection strategy to combine large seed and high protein, correlation between seed size and protein among the selections was studied. The earlier studies found no correlation between seed size and protein in pigeonpea germplasm (Dahiya & Brar, **1976**; Singh & Jambunathan, **1981**). On the contrary, Reddy et al. (**1979**), Saxena et al. (**1987**) and Bahl et al. (**1979**) reported negative associations between seed size and protein. Obala et al. (**2020**) also observed negative association between protein and yield and positive association of protein with seed size in three F_2 populations. Bahl et al. (**1979**) opined that a negative relationship between seed size and protein implied that seed increases in size were due to the deposition of an increased amounts of starch, altering the starch:protein ratio. The negative correlations reported between seed size and proteins in the above-referred studies were not strong and accounted for a little variation.

In the present study, the ranges for seed size (3.9–14.2 g/100 seeds) and protein (18.8% to 35.6%) were large, and the correlation

TABLE 3 Examples of single-plant selections for protein per cent in F_4, F_5 and F_6 generations and their performance in F_7 progeny rows.

				F_7 progeny		
Cross I.D.	Selection I.D. no.	F_5 sel.	F_6 sel.	Mean	% gain over ck.	100-seed wt. (g)
C1	F_4 515	28.5	29.3	29.5 ± 0.06	30.4	11.1
(18.8–35.6)[a]	Check	19.8	21.1	23.4		10.1
Prog. Mean	F_4 531	27.1	27.8	28.7 ± 0.50	27.0	10.4
	Check	19.9	21.1	22.4		10.5
C2	F_4 566	29.3	29.0	30.4 ± 0.38	31.6	9.0
(22.2–29.3)[a]	Check	19.8	21.6	23.8		10.5
	F_4 681	28.9	27.8	28.6 ± 0.78	35.7	7.9
	Check	18.8	20.8	22.1		10.6
C4	F_4 684	27.0	28.3	29.0 ± 0.76	33.4	6.9
(19.9–27.0)[a]	Check	17.8	20.8	23.1		10.3
	F_4 687	27.0	27.8	28.8 ± 0.33	35.5	7.3
	Check	17.8	20.8	22.3		10.6

Note: C1 = Baigani × *Cajanus scarabaeoides*, C2 = Pant A3 × *Cajanus albicans* and C4 = T21 × *Cajanus sericeus*.
[a]Range for protein (%) in F_4.

between these two traits was negative ($r = -.13^{**}$) but with a low reliability factor ($R^2 = 1.69\%$). These results (Table 4) suggested that in pigeonpea, unlike other legume and cereals, genetic improvements can be made simultaneously for seed size and protein.

This was validated from the seed size recorded in some high-protein lines derived from interspecific crosses. For example, the high-protein lines HPL 2, HPL 7, HPL 40 and HPL 51 have high (27% and 29%) protein content, and they have large seeds with their 100-seed weight range between 10.0 and 12.1 g.

6.4 | Transgressive segregation for protein content

In cross 'Baigani' × *C. scarabaeoides*, the intrapopulation variability for protein content was large (18.8% to 35.6%), and it extended the donor parental limit by a significant margin. The highest protein value recorded in a segregant was 35.6%, and this unique recombinant was 24.04%** greater in protein content than the donor species (28.4%). Such events, generally referred to as 'transgressive segregation' are facilitated by the divergence of parents and complementation of alleles with additive genetic actions and expressed in some rare recombinants of favourable alleles. Product-wise, such events could appear on positive, negative or both the sides of the performance curve. Durga (1989) reported that in pigeonpea, the genes controlling protein content are additive and/or complementary in nature. Rick and Smith (1953), Grant (1975) and Vega and Frey (1980) also opined that the generation of extreme variability in a population is a consequence of complementation of diverse alleles. They also mentioned that the emergence of transgressive segregants is associated with the presence of certain recessive complementary alleles whose expression is masked by the major/dominant alleles. These conclusions were further confirmed through marker-based QTL studies (de Vicente & Tanksley, 1993). Hence, in the present case, it is postulated that cv. 'Baigani' and the *C. scarabaeoides* carry different set of alleles for controlling protein; and their complementation produced the transgressive segregants. However, to understand their true genetic system in pigeonpea, some targeted genetic and molecular studies are needed.

7 | EVALUATION OF HIGH-PROTEIN SELECTIONS

Once transferring the high-protein trait from wild species to the cultivated types was successfully achieved, the next goal was to test the elite lines with respect to stability across environments, nutritional parameters, biological efficiency and finally the productivity. A brief account of these research activities is presented in the following text.

7.1 | Stability across diverse environments

To understand the effects of diverse environments on the protein content, four high-protein lines were evaluated at six diverse locations spread over six northern and southern provinces of India. At each location, the high-protein lines were significantly superior to the control cultivar (Table 5). Among the test lines, HPL 24 appeared to be the best; and its protein content ranged from 31.3% to 32.3% with a mean of 31.6%.

These observations indicated that high-protein lines retained their trait that was transferred from the wild species. From these data, it can be concluded that the protein trait is stable, and these high-protein lines are safe donors for breeding high-yielding high-protein cultivars.

7.2 | Nutritional evaluation of high-protein selections

Singh et al. (1990) studied nutritional profile of the high-protein inbred lines developed through selection. The protein content in the high-protein selections was significantly superior. In HPL 40 (31.1% protein), the superiority over control was 34.4%. Similarly, in HPL 8 (28.8% protein), the superiority over control was 24.4% (Table 6). This information suggested that the pedigree breeding method was highly successful in transferring the high-protein trait from wild relatives of pigeonpea to the cultivated types. As expected, the starch component in the high-protein lines was relatively less (54.3% to

TABLE 4 Estimates of correlation coefficient (*r*) between seed size and protein among the F_7 single plants.

		Seed size (g/100)		Protein (%)		
Cross	*N*	Range	Mean	Range	Mean	*r*
C1	1231	3.8–14.2	7.8	18.8–35.6	26.3	−.12**
C2	165	5.0–12.0	7.6	21.8–31.2	26.1	−.07
C3	268	4.5–10.7	6.9	22.2–30.4	26.4	−.07
C4	91	5.3–10.4	8.9	22.0–30.6	26.8	−.30**
C5	213	5.7–13.7	9.1	19.9–30.3	25.0	.28**
Total	1974	3.9–14.2	7.8	18.8–35.6	26.2	−.13**

Note: C1 = Baigani × *Cajanus scarabaeoides*, C2= Pant A3 × *Cajanus albicans*, C3 = Pant A2 × *C. scarabaeoides*, C4 = T21 × *Cajanus sericeus* and C5 = T21 × *C. scarabaeoides*.

**Significant correlation.

TABLE 5 Stability of protein content of four high-protein selections.

Location	Lat °N	HPL 24	HPL 25	HPL 26	HPL 28	Loc. mean	Control	SE (±)
Gulbarga	17.3	32.1	29.9	-	27.6	29.86	23.0	0.49
Patancheru	17.4	31.3	28.6	29.7	27.8	29.35	23.3	0.26
Jalna	19.8	32.2	28.9	29.7	30.4	30.30	23.1	0.69
SK Nagar	24.3	30.9	28.4	29.0	27.3	28.90	21.4	0.36
Gwalior	26.2	32.3	30.4	28.2	27.3	29.55	22.0	0.71
Hisar	29.1	31.1	29.6	31.7	29.2	30.40	24.5	0.51
Mean		31.65	29.30	29.66	28.33	-	22.88	
% gain over control		38.33	28.06	29.63	23.81	-		

Source: Singh et al. (1990).

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 HPL 40	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).

55.6%) than that of the control (58.7 to 59.3%). Also, the high-protein lines were found to be marginally low (2.5% to 2.6%) in their fat contents when compared with control cultivars (2.9% to 3.1%). The differences in major protein fractions of the high- and normal-protein lines were also large. In comparison with controls (60.3% to 60.5%), the globulin was also higher (63.5 to 66.2%) in the high-protein lines, and the reverse was true for glutelin.

7.3 | Biological evaluation of high-protein inbreds

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 with 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.

The biological evaluation of the test lines 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 sulphur-containing amino acids. They also concluded that whole seeds of high-protein lines for animals and *dal* for human beings are nutritionally beneficial; and such lines can help, if promoted appropriately, in addressing the issues related to rural nutrition. The evaluations of these high-protein lines revealed that per hectare, 350–450 kg crude protein can be harvested, reflecting an additional advantage of 80–100 kg protein/ha. Cultivation of these lines will markedly improve availability of protein to farmers without sacrificing seed yield. The results of rat feeding trials of these high-protein lines showed that cooked *dal* (splits) from the high- and normal-protein lines were similar in true protein digestibility, biological value and net protein utilization. It was concluded that the high-protein lines were nutritionally superior to normal-protein cultivars as the former contain quantitatively more utilizable protein and sulphur-containing amino acids.

7.4 | Assessment of productivity

In F_{10} generation, the first set of yield trials of high-protein (≥28%) lines was conducted. The results from evaluation were very encouraging and provide an opportunity to breed high-yielding high-protein pigeonpea cultivars. In the evaluation of non-determinate lines, the yield of the top two test lines (HPL 40-5 and HPL 40-17) was over 2 t/ha, and it was like that of the control BDN 1 (2.02 t/ha). These lines also compared well with control in maturity and 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 7). These results demonstrated that in pigeonpea seed yield, seed size and protein can be enhanced simultaneously. It is estimated that by growing such lines in 1 ha about 350–450 kg crude protein could be harvested, with an advantage of 80–100 kg protein/ha over the standard control.

Selections were made in this project in F_2 for both improved plant type and high protein and this continued up to F_8 generations. The protein content in five selected high-protein lines exceeded the control by 3.5% to 4.5%. In F_9, the advantage ranged between 1.7% and 5.5%. Interestingly, some advanced breeding lines approached the check yield but with high protein. The protein yield harvested from the control was 393 kg/ha, while the best high-protein line produced 468 kg protein/ha. Shalve (2019) reported the development of high-protein breeding lines of pea with 30% protein instead 20% in the control. Whan and Crosbie (1987) reported no success in breeding high-protein wheat and that the environment exerts a strong influence on protein content as that of yield.

8 | APPLICATION OF GENOMICS IN BREEDING HIGH-PROTEIN PIGEONPEA LINES

Pigeonpea and its wild relatives have $2n = 22$ chromosomes with a genome size of cultivated pigeonpea of 833.07 Mbp (Varshney et al., 2012). The wild relatives of pigeonpea are useful resources for the traits not found in primary gene pool. These include resistance/tolerance to drought, insects and high protein. Transferring these traits to the cultivated types is not only difficult, but it also suffers from low probability of success due to the presence of unwanted linkage drag. In this context, recently developed genomics resources in pigeonpea can prove a boon to breeders. Identification of traits associated molecular markers can guide breeders in selecting some rare recombinant events; and these will reduce the proportions of unwanted alleles in the new genetic background. In pigeonpea genomic resources have become available and a number of marker traits associations studies were conducted (Bohra et al., 2012; Bohra et al., 2020; Obala et al., 2019; Saxena et al., 2010); and this opened the door for the deployment of genomics-assisted breeding in pigeonpea. In order to assist breeding efforts for the development of pigeonpea cultivars with high seed protein content, a set of early generation populations (F_2s) were used to identify markers associated with seed protein content (Obala et al., 2019). Genome sequencing data together with phenotyping data identified sequence-based markers and associated candidate genes for seed protein. Furthermore, assay of 16 of the polymorphic CAPS markers on an F_2 population of a high- and low-protein cross resulted in identification of four markers which co-segregated with seed protein content. These four markers derived from mutations in four genes will be useful in breeding high-protein genotypes in pigeonpea. Moreover, new insights coming from genomics studies will pave the path of better handling of complex traits such as seed protein content in routine breeding programmes.

TABLE 7 Seed yield and protein harvest from high-protein lines at Patancheru.

Genotype	Maturity (days)	100-seed wt. (g)	Yield (t/ha)	Protein (%)	Protein yield (kg/ha)
Non-determinate selections					
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
Determinate selections					
HPL 8-10	163	10.5	1.66	26.5	353
HPL 8-16	162	10.5	1.57	27.4	344
ICPL 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).

9 | CONCLUSIONS

Pigeonpea, with 20% to 22% protein content, is a primary source of digestible protein for millions in various developing nations of South Asia and South and East Africa. The protein content in the primary gene pool is limited; therefore, some high-protein donors were searched among its crossable wild relatives. These were used for the genetic enhancement of seed protein in pigeonpea; and a well-planned scheme was launched to identify specific high-protein donor accessions, develop their hybridization, selection and integrated laboratory assessments programmes. The breeders were able to increase the seed protein to about 28% with no yield penalty. The high-protein lines had good morphological and nutritional grain qualities with no antinutritional factors involved. Also, their biological efficiency matched well with popular pigeonpea cultivars. Such lines if promoted properly can help in the nutritional security of rural masses of Asia and Africa.

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CONFLICT OF INTEREST STATEMENT

Authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

KBS conceived the idea. KBS and RKS developed and edited MS. LJR and KBS organised the field trials, collected, and analysed the data.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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