
Wide Crossing Technology for Pigeonpea Improvement

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Nalini Mallikarjuna, Rachit K. Saxena, M. Byre Gowda
and Rajeev K. Varshney

Abstract

Pigeonpea (*Cajanus cajan* Millsp.) has ample genetic and genomic information now. It is endowed with rich germplasm in different gene pools. One of the easiest material to use in those are in the primary gene pool, which are closely related to cultivated pigeonpea. It is observed that species placed beyond the primary gene pool are a rich source of genetic variation. They contribute beneficial traits to pigeonpea such as pest or disease resistance, resistance to abiotic stresses, cytoplasmic male sterile systems (CMS) leading to yield improvement, and some novel traits such as homozygous pigeonpea lines. To effectively utilize the immense variation present in the secondary, tertiary, and quaternary gene pool of pigeonpea, a thorough knowledge of crossability and concerted effort is essential.

4.1 Introduction

Genetic variability is the foundation for any breeding program, and breeders look for useful traits first within the primary gene pool and if unsuccessful, then look for the genes available within wild relative of the crop species. These wild species, crossable or non-crossable, may carry genes which may have been lost during natural selection and/or breeding for key economic traits. Mining and utilization of the required trait(s) from wild species is resource intensive, and its success depends on the individual breeder's knowledge, efforts and resources. There are many examples where genes for diseases, insect, quality, etc., have been identified and used in breeding for stability

N. Mallikarjuna · R.K. Saxena · R.K. Varshney
International Crops Research Institute for the
Semi-Arid Tropics, Patancheru 502324, India

N. Mallikarjuna (✉)
#213, Esha Krupa, 9th Cross 3rd Stage, Gokulam,
Mysore, Karnataka, India
e-mail: mysorenalinicgiar@yahoo.com

M. Byre Gowda
All India Co-ordinated Research Project on
Pigeonpea, University of Agricultural Sciences,
GKVK, Bengaluru, Karnataka, India

and developing new genetic system such as male sterility. The development of technologies such as transformation, somatic hybridization, tissue/embryo culture has made it possible to utilize genes from the wild species separated by vast hybridization barriers.

In recent time, the change in agricultural environment is making subsistence agriculture more difficult and unpredictable. To overcome such threats as drought, high temperature, and emergence of new biotypes of insects and diseases necessitates the search of new genes from the germplasm. The wild species have greater probability of harboring the genes which can sustain the climate changes because over the time they have survived under diverse environments and may carry special survival mechanisms, not available among the cultivated types. Pigeonpea belongs to the subtribe *Cajaninae* which contains 13 genera. The earlier taxonomists considered genus *Atylosia* and *Cajanus* different but closely related. Subsequently, van der Maesen (1980) merged the two genera together and identified as genus *Cajanus*. At present therefore, this genus contains 32 species. Of these, 18 are endemic to Asia, 13 to Australia, and one to Western Africa. The other related genera are *Rhynchosia*, *Dunbaria*, *Flemingia*, *Paracalyx*, *Eriosema*, *Adenodolichos*, *Bolusafra*, *Carissoa*, *Chrysoscias*, and *Baukea* (van der Maesen 1986).

Pigeonpea originated about 3500 years ago, and its landraces contain a lot of variability (Remanadan 1990), but the recent genomics studies showed lack of genetic diversity within primary gene pool, and this is a matter of concern to breeders engaged in the genetic improvement of the crop. Hence, the viable option appears to be the utilization of its wild relatives from secondary, tertiary, and quaternary gene pools using appropriate gene transfer techniques.

4.2 Primary Gene Pool

Considerable progress has been made in pigeonpea improvement by using variability within the cultivated species. In spite of the large germplasm collection in the primary gene pool, it

is not widely used (Wright 1997) as information on the presence of useful traits is not easily available and necessitating an extended period of research whenever utilized (Goodman 1990). To overcome these issues, core and mini core collections have been developed (Upadhyaya et al. 2006). Variation within the primary gene pool is of importance as they are easy to use with quicker gains and can be directly released as cultivars. Progress has been made in the utilization of material from the primary gene pool. Varieties BDN-1 and Maruthi are selections from pure-line breeding which are popular even today (Bantilan and Joshi 1996). Development of high yielding varieties such as ICPL 87, ICPL 151, Prabhat, T 21, Pusa Ageti, CO 5, and JA 3 has also been reported (Singh et al. 2005). In pigeonpea, 57 ancestors were used to develop 47 varieties. The top 10 ancestors contributed 48% to the genetic base of the released varieties (Kumar et al. 2003), thus narrowing the genetic base of the crop.

4.3 Secondary Gene Pool

Compatible wild relatives of pigeonpea which are placed in the secondary gene pool do not need specialized techniques in the crossability experiments in majority of the cases, with a few exceptions (Mallikarjuna et al. 2011a).

4.3.1 Cytoplasmic Male Sterile Systems

Cytoplasmic male sterile systems were developed for pigeonpea exploiting the cross-pollination mechanism and utilizing wild *Cajanus* species. So far, nine CMS systems have been reported utilizing wild relatives of pigeonpea (Mallikarjuna et al. 2012; Srikanth et al. 2015). Of these, seven have been developed utilizing wild relatives from secondary gene pool as the female parent. Two systems have cultivated pigeonpea cytoplasm with the utilization of wild species as the male parent (Mallikarjuna and Saxena 2005; Srikanth et al. 2015).

4.3.2 Cleistogamy

Insect-aided natural outcrossing in pigeonpea is a universal event (Saxena et al. 2016), and it leads to rapid contamination of pure lines. The breeders were on the lookout for a genetic trait that could protect the genetic purity at no cost basis. Saxena et al. (1992) discovered a floral modification that inhibits cross-pollination under open fields. This trait, selected from the cross *C. cajan* × *C. lineatus* and identified as “partially cleistogamous” flower, restricts natural outcrossing to less than 1%. This trait is controlled by a single recessive gene and thus easy to handle in pedigree breeding programs. Its stability across the environments makes it an ideal trait for incorporation in future cultivars.

4.3.3 High Protein

High protein breeding lines were developed from *C. sericeus*, *C. albicans*, and *C. scarabaeoides*. Significant positive correlation between seed size and protein content was observed in lines derived from *C. scarabaeoides*. Lines HPL 2, HPL 7, HPL 40, and HPL 51 are some of the high protein and high seed weight lines derived from wild species (Saxena et al. 1987). More recently, crosses between pigeonpea and *C. acutifolius* yielded progeny with high seed weight. High seed weight accompanied by beige seed color is a desirable trait (Jadhav et al. 2012).

4.3.4 Insect Resistance

C. acutifolius, a wild relative from secondary gene pool and native of Australia, can be crossed with pigeonpea as a one-way cross. The reciprocal cross using *C. acutifolius* as the female parent aborts to give rise to immature seeds. In vitro, interventions are necessary to obtain hybrid plants (Mallikarjuna and Saxena 2002). Advanced generation population from the cross utilizing *C. acutifolius* as the pollen parent has shown resistance for pod borer damage

(Mallikarjuna et al. 1997; Jadhav et al. 2012), variation for seed color, and high seed weight. Some of the lines showed high level of resistance to pod borers, pod fly, and bruchid under unprotected field conditions (Jadhav et al. 2012). Bruchid resistance (Jadhav et al. 2012) is an important trait for pigeonpea seeds under storage as resistance to the pest has not been observed in cultivated pigeonpea.

Another species from secondary gene pool, namely *C. lanceolatus*, was crossed successfully with cultivated pigeonpea at ICRISAT and progeny lines developed (Srikanth et al. 2013). F₁ hybrids flowered but some of the hybrids were pollen sterile, and in the rest of the hybrids, pollen fertility varied from 25 to 55%. All the hybrids were female fertile. Progeny lines developed from the cross were screened for bruchid resistance. Bruchid growth and survival was inhibited in the lines derived from *C. lanceolatus*. Some of the lines showed delayed bruchid growth and delayed life cycle thus showing antibiosis mechanism of resistance to bruchids. Lines were screened for protein content, and some of the lines showed higher protein content than both their parents. Further, biochemical analysis showed higher content of proteinase inhibitor activity in some of the lines (Srikanth et al. 2017). Previously, Satishkumar (1985) had attempted crossing pigeonpea with *C. lanceolatus* but obtained sterile hybrids which did not flower and remained in the vegetative stage.

4.3.5 Water Logging

Some of the advance generation lines derived from *C. acutifolius* were screened for water logging by germinating them and later growing them under water logged conditions. A few lines grew under water logged conditions, and formation of lenticels was observed in the region above the water surface. The region gave rise to roots which entered the soil through the water surface. This shows that some of these lines may survive water logged conditions as seen in some pigeonpea lines (Hingane et al. 2015).

4.4 Tertiary Gene Pool

There are 20 wild species in the tertiary gene pool of pigeonpea (Mallikarjuna et al. 2011a). Until now, two wild *Cajanus* species from this gene pool have been successfully crossed and traits of interest transferred (Mallikarjuna et al. 2011b). *Cajanus platycarpus* was successfully crossed utilizing hormone-aided pollinations and in vitro interventions (Mallikarjuna et al. 2011a) to obtain hybrids. Progeny lines showed variation for days to flower, growth habit, seed weight and number, seed color, resistance to pod borer, pod fly, bruchids, fusarium, and sterility mosaic disease and CMS (Mallikarjuna et al. 2011b, 2012). Some chasmogamous lines (Cherian et al., 2006) were identified in CMS lines, a trait favoring total cross-pollination. Hence, utilizing *C. platycarpus* not only broadened the genetic base of pigeonpea but it was possible to introgress useful traits.

More recently, another species from tertiary gene pool, namely *C. volubilis*, was crossed with pigeonpea (Mallikarjuna et al. 2014). In F₂ generation, extra short duration lines were recovered in 2011. These lines flowered earlier than the short duration cultivar ICPL 85010 which was the female parent of the cross. Dwarf growth habit coupled with determinate and semi-determinate plants was observed. In the determinate types, the number of pods per inflorescence and the number of inflorescence was more than that observed in the extra-early and determinate cultivar MN5. Allele-specific marker assay developed for the SNP (T/A) was used to genotype 21 F₂ progeny derived from *C. volubilis*. Two bands (one common band (848 bp) and one allele-specific band (734 bp/167 bp) appeared in all the samples. The degenerated common primers (TFL1_PCR_CF and TFL1_PCR_CR) amplified 848 bp-specific fragment, specific for determinate types, among all genotypes (Mir et al. 2014). As short duration is a desirable trait in pigeonpea improvement, and there is an emphasis to look for this trait while breeding for better pigeonpea especially for higher altitudes and latitudes. Although early flowering with short stature is available in

cultivated pigeonpea germplasm, the source identified in *C. volubilis* derivatives may have a different genetic background, as *C. volubilis* is a tertiary gene pool species.

4.5 Quaternary Gene Pool

There are 11 related genera, namely *Rhynchosia*, *Flemingia*, *Dunbaria*, *Erisema*, *Paracalyx*, *Adenodolichos*, *Bolusafra*, *Carissoa*, *Chrysocias*, and *Baukea* including *Cajanus* under the subtribe Cajaninae. Many of these genera are classified as underexploited legumes. *Rhynchosia* is one such example as it harbors important nutritional and therapeutic properties (Drabu et al. 2011), with the presence of phytochemicals such as alkaloids, glycosides, anthraquinones, carotenoids, coumarins, dihydrochalcones, fatty acids, flavonoids, steroids and triterpenoids (Bakshu and Venkataraju 2001). Some species of *Rhynchosia* are used in human and animal diet (Oke et al. 1995). Many of the tribal communities in India soak the seeds in water and consume the seeds after boiling and decanting many times to get rid of unwanted constituents (Murthy and Emmanuel 2011). Many of the *Rhynchosia* species are known to exhibit antitumor and thus curative properties. Normally, during cancer treatment iron deficiency and anemia are major issues. It was observed that treatment with *Rhynchosia* seeds restored hemoglobin (Hb) count, RBC and WBC count to normal levels. These traits are also important in the treatment of dengue fever, an ailment caused by a variety of mosquito bite. With the interest in dietary flavonoids, suppression of cancer, and treatment in dengue fever, progeny lines developed from pigeonpea and *Rhynchosia* cross would be an asset in pigeonpea improvement.

None of the genera in the quaternary gene pool have been successfully crossed with pigeonpea until now. Among the genera in the quaternary gene pool, *Rhynchosia* was selected to initiate crossing/introgression/gene transfer experiments as it had many desirable properties as listed above. It was possible to successfully cross *Rhynchosia* with pigeonpea through

hormone-aided pollinations (Mallikarjuna et al. 2014). The success rate of crossing *Rhynchosia* was low not exceeding 1–2%, but it was possible to obtain hybrids. Screening the hybrids with molecular markers confirmed the hybridity (Mallikarjuna et al. 2014). Although the initial process of crossing *Rhynchosia* with pigeonpea was challenging, nevertheless, hybrids were obtained. They were fertile, and it was possible to obtain self and backcross progenies. Experiments to screen and study the progeny lines for different traits/constraints are in progress.

4.6 Diversity in Expression of Traits Due to Alien Introgression

4.6.1 Segregation of Traits Following Mendelian Pattern of Inheritance

Wild relatives from secondary gene pool cross with cultigen following the Mendelian pattern of inheritance. This meant the F_1 produced, broadly showed the expression of both the parental species in the ratio of 1:1. When the F_1 was backcrossed the recurrent parent, the progeny lines segregated to traits in the ratio of 3:1, again following the Mendelian pattern of segregation. Similar results were observed when a tertiary gene pool species *C. platycarpus* was crossed with the cultigen (Mallikarjuna et al. 2011b) DArT analysis of the BC4F1 lines, where the recurrent parent was the cultigen, showed that it followed the Mendelian pattern of inheritance in having approx. 94% *C. cajan* genome/DNA, and the remaining was that of *C. platycarpus* (Mallikarjuna et al. 2011a).

4.6.2 Segregation Pattern Showing Genomic Dominance

Cajanus volubilis, a wild relative in the tertiary gene pool of pigeonpea, produced F_1 hybrids when crossed with the cultigen (Mallikarjuna et al. 2014). The F_1 hybrid showed traits of both

the parental species, i.e., of both *C. volubilis* and the cultigen, and the hybridity was further confirmed by molecular SSR analysis (Mallikarjuna et al. 2014). F_1 hybrid was selfed to produce F_2 progeny lines. All the plants from F_2 onward showed genomic disequilibrium by resembling the cultigen with respect to morphological traits. None of the plants had any morphological traits or characters of *C. volubilis*. Molecular analysis of F_2 plants confirmed that they resembled the cultigen. Such a phenomenon has not been observed in pigeonpea-wide crosses using compatible wild relatives from secondary gene pool (Mallikarjuna and Saxena 2002). Genomic asymmetry was observed in the present cross as distantly related genomes of *Cajanus*, i.e., of the cultigen and that of *C. volubilis*, were brought together for the first time through hybridization. Bringing together distantly related genomes involves radical and rapid mode of speciation by means of interspecific hybridization. It has been observed in wheat when different genomes were brought together there was predominance of one genome over the other (Flagel et al. 2009; Rapp et al. 2009). In wheat A and B genome hybrids, B genome exhibited more or higher marker polymorphism than the A genome (Chao et al. 1989). Genome-wide transcriptome analysis in synthetic *Arabidopsis* allotetraploids showed that expression patterns from one genome could be dominant over the other (Wang et al. 2006a, 2006b). Pumphery et al. (2009) observed that some of the genes were similar to one of the parental genomes in synthetic hexaploid wheat. In *C. volubilis* progeny lines too, predominant expression of one of the parental genome over the other was observed and this can be explained taking examples of wheat, *Arabidopsis*, etc., that when distantly related genomes are brought together, there is silencing of the expression of the other genome completely or partially, although the DNA from the other parent is present, at least for many of the morphological traits, female parental genome expression is obvious.

Genome asymmetry has been observed not only for the expression of morphological traits but for other important traits, in a few crops when

different genomes are brought together by hybridization. Genome asymmetry in the control of storage proteins has been observed in wheat (Levy et al. 1988). In *Gossypium hirsutum*, genome asymmetry was found in the accumulation of seed storage proteins (Hu et al. 2011). Genome asymmetry in the control of agronomic, disease, and pest resistance traits has also been observed in wheat (Feldman et al. 2012).

There were unsuccessful attempts in the past to cross pigeonpea with *C. volubilis* pollen (Pundir and Singh 1985). This is the first report of successful interspecific hybridization between pigeonpea and *C. volubilis*. Morphological traits of the F₁ hybrid were more skewed toward the female parent, and such phenomenon is not new when distant genomes are made to come together through wide hybridization. Molecular analysis confirmed its hybridity. *C. volubilis* is a wild relative of pigeonpea placed in its tertiary gene pool (Mallikarjuna et al. 2011b; Bohra et al. 2010). Genomic studies have also shown its distant relationship with cultivated pigeonpea (Pangaluri et al. 2007). Genome-wide transcription analysis in synthetic *Arabidopsis* allotetraploids showed that expression patterns from one genome could be dominant over the other genome (Wang et al. 2006a, 2006b). Pumphery et al. (2009) found that a small percentage of hybrids between wheat and synthetic hexaploids was similar to one of the parents. We report for the first time in pigeonpea that such a phenomenon is taking place in the hybrid between *C. cajan* (cultivated pigeonpea) and *C. volubilis* with the morphology of the F₂ hybrids skewed toward the female parent. Genetic control in storage proteins has been observed in allopolyploid wheat. Galili and Feldman (1984) showed that inactivation of endosperm protein is brought about by an inter-genomic suppression. Wheat genome-driven control of some agronomic, pest, and disease resistance was observed in wheat. Peng et al. (2000) observed that R-gene cluster in the B genome of wheat and high marker clustering in the B genome than the A genome is the result of expression of genome asymmetry. The explanation for ability of one genome to suppress the activity of genes in another in newly formed

hybrids with different genomes may be to prevent defective organ formation/phenotype. This may be a protective mechanism to obtain viable plants.

4.7 Development of Homozygous Lines

4.7.1 Tissue Culture

Haploids are plants that contain gametic number (n) of chromosomes. Haploids and consequent production of “double haploids” (DH), obtained by doubling of chromosomes either spontaneously or induced via chemical methods, have since been applied to many crop improvement programs, and protocols are available for more than 200 plant species (Bhojwani and Razdan 1996). DH is the fastest route to obtaining homozygosity and can be developed in a single laboratory-based generation. They are equivalent to inbred lines developed by conventional breeding program requiring 6–7 generations of selfing to achieve satisfactory level of homozygosity (Mallikarjuna et al. 2005). Owing to their homozygous and true breeding nature, they are of importance in plant breeding. But legumes are considered to be recalcitrant to DH production with a few successful examples such as *Medicago truncatula* (Lanas et al. 2006), *Glycine max* (Moraes et al. 2004), *Pisum sativum* (Ochatt et al. 2009), and *Lupinus angustifolius* (Kozak et al. 2012).

Except for one report (Kaur and Bhalla 1998), haploid/doubled haploids have not been reported in pigeonpea in spite of multiple and concerted efforts later on. Homozygous or pure lines have gene sets which are exactly identical or two identical haploid genomes. Homozygous lines or doubled haploid plants once developed will be of great significance in pigeonpea, which is a partially cross-pollinated crop. Cross-pollination induces variability which is a desirable trait in pigeonpea, but not always. In certain breeding experiments such as CMS (cytoplasmic male sterility), variability or heterozygosity is not desirable in the development of restorers and to

exploit heterosis. Homozygosity can be achieved by anther culture, but this technology does not work for pigeonpea in spite of concerted and repeated efforts (Croser et al. 2006).

4.7.2 Wide Crosses

Utilizing related genera *Rhynchosia*, it has been possible to obtain hybrids between *Rhynchosia* species and cultivated pigeonpea (Mallikarjuna et al. 2014). Although the F₁ was a true hybrid, subsequent generation progenies resembled pigeonpea. Molecular analysis of F₂ progeny lines revealed complete homozygosity for all the loci tested. Hence, the resultant plants were completely homozygous. In 2012 field evaluation, F₄ progeny lines showed complete resemblance to the female parent, i.e., to cultivated pigeonpea. Many distantly related species are difficult to cross and when forced to cross, get their genome eliminated. Although this has not been reported for any legume species, in cereal crops such as barley, homozygous plants are obtained in one step by crossing barley with *Hordeum bulbosum*. The method called the bulbosum technique, first reported by Kasha and Kao (1970), is now a routine technique of haploid production in barley through chromosome elimination by wide cross technology. The chromosome elimination phenomenon is quite prevalent among wide crosses between wheat and *H. bulbosum* as well (Barclay 1975). An added advantage in such cases is that there is spontaneous doubling of female parental chromosomes as observed in barley and in pigeonpea too. Mallikarjuna et al. (2005) reported multicellular microspores in most of the anther in a chickpea wide cross *C. arietinum* × *C. pinnatifidum*. If such anthers/microspores are cultured, it may be possible to obtain haploid plants in chickpea. This could be yet another method of haploid production through wide cross technology.

Homozygous plants offer a promising alternative to recurrent selfing for many years/generations, for rapid inbred line development. Even if the inbred lines are developed after

repeated selfing, it is known that certain regions of the genome retain heterozygosity (Nair et al. 1995). In none of the leguminous crops, this phenomenon of homozygosity through wide crosses has been reported.

Normally, a diploid will have a combination of different haploid genome/s obtained from each parent through gametic fusion in sexual reproduction. When homozygous lines are developed through wide hybridization, there is preferential elimination of the paternal genome and doubling of the maternal genome chromosomes, thus creating 100% homozygosity in one step. Dominance is eliminated in homozygosity, and much less progeny is needed in the study. For example, for two desired genes, using homozygous lines, one needs to grow four homozygous lines instead of growing 16 when selfed to obtain desired genotype. For four desired genes, using homozygous route, one would need to grow 16 homozygous lines, instead of growing 256 through the selfing route.

4.8 Conclusion

No other food legume crop has been investigated for alien introgression and succeeded in crossing wild relatives from all the gene pools, namely secondary, tertiary, and quaternary gene pools. Pigeonpea is one crop where tremendous progress has been made to cross wild *Cajanus* species from different gene pools and introgress genes/traits successfully. With these successes, it can no more have a narrow genetic base. With the advances in pigeonpea genomics, and a major effort in sequencing the crop, and success in wide crosses in pigeonpea, it has emerged from being labeled as an orphan crop to a trend setter. Recent successes in wide crosses show that it is possible to introduce desirable traits such as pod borer resistance, develop CMS systems, develop lines with multiple disease and pest resistance, change plant type, and increase seed weight and yield. An added advantage is the availability of homozygous lines for any applied research.

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