Euphytica (2010) 173:329–335
DOI 10.1007/s10681-009-0088-0

Insect-aided natural out-crossing in four wild relatives of pigeonpea

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Received: 4 June 2009 / Accepted: 18 November 2009 / Published online: 2 December 2009

Abstract Insect-aided natural out-crossing in pigeonpea [*Cajanus cajan* (L.) Millsp.] is a common event. It is considered to be a prime constraint in maintaining genetic purity of cultivars and genetic stocks. On the contrary, the out-crossing has also been exploited to select high-yielding varieties from landraces. This paper, for the first time, reports natural out-crossing in four wild relatives of pigeonpea. The highest (17.1%) natural out-crossing was recorded in *C. lineatus* and it was comparable to the control cultivar Asha (22.2%). *C. albicans* and *C. scarabaeoides* exhibited 10.0 and 8.3% out-crossing, respectively. *C. sericeus* was found to have lowest (2.3%) natural out-crossing. A process of breeding a new cytoplasmic-nuclear male-sterility (CMS) system arising from a natural hybrid of *C. lineatus* has also been described. Once stabilized, this CMS will be used in breeding programs aimed to develop high-yielding pigeonpea hybrids.

Keywords Natural out-crossing · Pigeonpea · [*Cajanus cajan* (L.) Millsp.] · Natural hybrids · Male-sterility · Wild relatives

Introduction

The present day domesticated plant species have evolved over centuries from their ancestors through various natural events such as gene mutation, hybridization, and selection for survival and adaptation to specific environments. Such natural forces not only generate new variability but also lead to stabilization of diverse selections that sometimes result in the establishment of a new species. Such species generally contain genes for some economically important traits that are now missing in the domesticated types and harbor from generations. Harlan and de Wet (1971) classified the vast intra-generic variation on the basis of their ability to exchange the genetic materials from one species with the other into the primary (cultivated germplasm), secondary (wild species which can be crossed easily with cultivated types), and tertiary (wild relatives of the species which either cannot be crossed with the cultivated types or need special techniques such as hormone treatment or rescue of hybridized embryo) gene pools. Pigeonpea belongs to the genus *Cajanus* within *Leguminosae* family. Earlier the genus *Cajanus* was considered as a monotypic genus with a single species and another genus *Atylosia,* where most wild relatives are grouped, was the most closely related genera (De 1974). van der Maesen (1986) after studying various taxanomical details merged the genus *Atylosia* into *Cajanus*. Now the genus *Cajanus* embraces 30 wild species which vary considerably

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for their genetic affinity (Pundir and Singh 1985; Dundas 1990) to the cultivated species. Among these, 10 species are classified in the secondary gene pool while the remaining 20 species constitute the tertiary gene pool (Ramanadam 1990).

Unlike most legumes, pigeonpea flowers are prone to natural out-crossing and for this reason, it is considered as a partially cross-pollinated species. Howard et al. (1919) were the first to report natural out-crossing in pigeonpea. Bhatia et al. (1981) reported 24% natural out-crossing in pigeonpea at Patancheru. The out-crossing in this crop is mediated by a variety of insects (Onim 1981) and wind does not play any role in this event (Kumar and Saxena 2001). At Patancheru, the authors observed that besides cultivated pigeonpea, a number of insect species also regularly visited the wild relatives of pigeonpea and sometimes a few naturally out-crossed plants with distinct traits were also present. This paper contains the first report of natural out-crossing in four wild species of genus *Cajanus*. Also, some potential benefits of natural out-crossing have been described with suitable examples.

Materials and methods

Four wild relatives of pigeonpea, *Cajanus scarabaeoides*, *C. albicans, C. sericeus*, and *C. lineatus* representing secondary gene pool of genus *Cajanus* were selected for this study. Some important morphological traits (Fig. 1) of these species are briefly described below:

(i) *Cajanus sericeus* (Benth.ex. Bak) van der Maesen comb. nov., is an erect shrub. Its accession ICPW 162 (EC 121208) that was used in this study originated in Australia. Its leaves are trifoliate and leaflets are soft coriaceous, glandular on both sides, white-hairy, oblanceolate and narrow. Its pale yellow flowers with small pedicle are borne in bunches of 1–3 flowers in leaf axils along the main stem and primary branches. (ii) *C. albicans* (W. & A.) van der Maesen comb. nov., a perennial climber with a woody base, is endemic in hilly terraces at altitudes of 500–1,700 m and its accession ICPW 13 (JM 2337) was collected from Karnataka, India. The branches of *C. albicans* are long and green with whitish pubescence. Flowers are yellow with reddish tinge on external side of standard petal. Bracts are minute with pubescence. Pods are oblong, about 2–4 cm long, and densely covered with short hairs. (iii) *C. lineatus* (W. & A.) van der Maesen is an erect shrub, 2.5 m tall species with open growth habit. The accession used in this study (ICPW 42) was collected as NKR 76 from Karnataka, India. Its branches are erect with lanceolate and narrow leaves. The leaflets are soft, glandular on both sides, pubescent trifoliate, and obovate. Flowers are yellow and the calyx is pubescent. Pods are small, oblong, 1.2 cm long, and hairy. (iv) *C. scarabaeoides* (L.) Thouars is a small, perennial, woody trailing bush found in abundance in dry areas. The accession selected for this study (ICPW 89) was collected as JM 2865 from Himachal Pradesh in India. *C. scarabaeoides* can be observed trailing or climbing on grasses and shrubs. Its leaves are pinnately trifoliate, leaflets are obovate and glandular punctuate. The flowers are yellow with dorsally red veins. The pods are small and oblong which exhibits mechanical resistance and antibiosis to *Helicoverpa* pod borers.

Pigeonpea line ICP 5529, characterized with recessive obtuse leaves, was used as a control. The seeds of all the wild species genotypes and the control cultivar were collected from the gene bank of ICRISAT. To facilitate germination of the wild species their hard seed coat was scarified with a sharp blade. For sowing of these materials four rows of 4 m length were sown in a Vertisols with inter- and intra-row spacing of 150 and 50 cm, respectively. The plots selected for sowing the wild species were located in the middle of a pigeonpea field planted with diverse breeding materials during the rainy season 2006. All the recommended cultural practices were followed to raise a normal crop. From each accession the bulk seed was harvested at maturity. In the subsequent season, the open-pollinated seed was planted and within each bulk, counts were made for the parental and off-type plants. Using this data, the extent of natural out-crossing in each wild species was determined as the proportion of the off-type plants and expressed as percentage. Also morphological traits including growth habit, plant spread, leaf shape, pod shape, seed shape, and seed color were recorded in the wild species and their natural hybrid plants. For making hand pollinations, young buds of wild species were emasculated and immediately pollinated with the fresh pollen of the variety ICPL 87119 and after 3 weeks

Fig. 1 Plants of wild species (*left*) and their out-crosses (*right*)

counts were made for pod set. The pollen sterility of plants in F_1 and various backcross populations was determined by sampling 2–3 buds from each plant and crushing their anthers in the aceto-carmine solution. In each slide two microscopic fields were examined under a light microscope and counts were made for fertile (stained) and sterile (non-stained) pollen grains.

Results and discussion

The pollination biology of pigeonpea is interesting because, in spite of having typical leguminous flowers, the *Cajanus cajan* is not a truly self-pollinating species and a considerable degree of natural out-crossing occurs (Saxena et al. 1990). The large red and yellow flowers of this species contain nectar which attracts a variety of insects. In the present study it was observed that the pollinating insects frequently visited both pigeonpea and wild species and the peak pollinator activity was achieved between 11.00 and 15.00 h.

The majority of insects that visited the flowers of pigeonpea and its wild relatives belonged to *Hymenoptera* group and among these, *Megachile bicolor* (Fab.) and *Megachile conjuneta* Smith were the main pollinators. Surprisingly, the frequency of other *Megachilidae* pollinators such as *Apis dorsata* (Fab.) and *Apis florae* (Fab.) was considerably low. Bumble bees, *Xylocopa* spp. were also common in pigeonpea fields at Patancheru. Williams (1977) reported that at Patancheru, the *Hymenoptera* group of insects carried many fold more of pigeonpea pollen than *Diptera, Lepidoptera, or Homoptera*. The cross-pollination took place when the pollen-loaded insects sat on other un-pollinated fresh flowers and during foraging the stigmatic surface was rubbed against their body. Therefore, the extent of natural out-crossing primarily depended on the number of pollinating insects foraging in a field and for this reason, the natural out-crossing was found to vary considerably from one place to another (Saxena et al. 1990).

In the present study data related to frequency and duration of individual insect visits were not recorded but visual observations showed that on both control pigeonpea as well as the wild species (except *C. sericeus*), the number of insect visitations were more or less similar. In *C. sericeus*, such visits were shorter and less frequent. This could partly be due to its different canopy and flower structure and orientation of flowers on the plant. A perusal of *C. sericeus* plant showed that its flowers were small with a short (about 5 mm) pedicle while other wild species had longer (about 15 mm) pedicle and the flowers were relatively large. Further, the flowers of *C. sericeus* were placed in leaf axils on the main stem and primary branches therefore a major proportion of floral surface remains covered under crowded leaves (Fig. 1, insert). Perhaps such a floral orientation failed to attract more number of pollinators due to the poor visibility. On the contrary, the flowers of *C. lineatus* and *C. cajan* were well exposed at the top of plant canopy and thereby provided more opportunities for insect visitations and cross-pollination.

Extent of natural out-crossing

The proportion of off-type plants recorded within each species was used to estimate the extent of natural out-crossing that occurred in the preceding generation. Since this method of estimating out-crossing did not take into account the out-crossing taking place in the reverse direction (recessive to dominant), it estimated only half of the real out-crossing events. Onim (1981), therefore, suggested to multiply the observed number of out-crossed plants by a factor of ‘2’ to get a real out-crossing value in a particular set of materials or environment.

In the present study a large variation among different *Cajanus* species was recorded for one way natural out-crossing. The highest natural out-crossing (17.1%) was recorded in *C. lineatus* (Table 1) and it was comparable to the control pigeonpea genotype (22.2%). *C. albicans* exhibited 10.0% out-crossing and the extent of natural out-crossing in *C. scarabaeoides* was 8.3%. *C. sericeus* was found to have the lowest (2.3%) natural out-crossing. To rule out any inherent reason for the variation in natural out-crossing among the species, each accession was hand pollinated with the pigeonpea line ICPL 87119. The extent of pod setting following hand pollinations was good in all the four wild species (Table 1). The pod setting by hand pollination in *C. sericeus* was high (31%) but the natural out-crossing was low (2.3%), suggesting that there was no major crossability barrier and the low out-crossing was the consequence of non-preference of insect pollinators. The low pod set values for artificial and natural hybridizations in *C. scarabaeoides* were due to excessive flower drop, inherently associated with this species. (L.J. Reddy, unpublished data). Since natural out-crossing is primarily controlled by insect population, the out-crossing in the wild species may not be similar at different places.

Individual plant vigor, growth habit, and plant type are the main features that can be used to identify the hybrid plants with ease in the population of parental wild species. In general, the hybrid plants were vigorous with large biomass. Since non-determinate growth habit is dominant over determinate type, the natural hybrids in *C. sericeus* and *C. lineatus* were non-determinate. Similarly, other recessive traits such as ‘compact’ and ‘creeping’ plant types (Pundir and Singh 1985; Saxena and Sharma 1990) were absent in

Table 1 Natural out-crossing observed in four wild relatives of pigeonpea at Patancheru

Species	Accession	Plants harvested in 2006	Plants grown in 2007	Hybrid plants observed	Out-crossing (%)	Hand pollinations made	Pod set (%)
C. sericeus	ICPW 162	32	128	3	2.3	32	31
C. scarabaeoides	ICPW 89	28	120	10	8.3	25	8
C. albican	ICPW 13	29	120	12	10.0	18	16
C. lineatus	ICPW 42	36	123	21	17.1	55	29
C. cajan (control)	ICP 5529	34	126	28	22.2	100	29

Table 2 Some characteristics of normal and out-crossed hybrid plants of four wild relatives of pigeonpea

	C. sericeus		*C. albicans*		*C. lineatus*		*C. scarabaeoides*	
	Wild spp.	F_1 hybrid	Wild spp.	F_1 hybrid	Wild spp.	F_1 hybrid	Wild spp.	F_1 hybrid
Growth habit	DT	NDT	NDT	NDT	DT	NDT	NDT	NDT
Plant type	Compact	Spreading	Creeping	Spreading	Spreading	Spreading	Creeper	Spreading
Leaf shape	Oblanceolate	Lanceolate	Sub orbicular	Lanceolate	Lanceolate	Lanceolate	Oblanceolate	Lanceolate
Pod shape	Cylindrical	Flat	Flat	Flat	Flat	Flat	Flat	Flat
Seed shape	Flat square	Round	Flat	Round	Flat	Round	Orbicular	Round
Seed color	Gray	Gray	Brown	Brown	Gray	Gray	Black	Black
Plant width (cm)	50	75	–	170	67	190	50	85
Primary branches (no.)	11	15	5	6	12	5	4	8
Branch length (cm)	31	82	65	128	53	72	63	98
Leaf area (mm^2)	15.0	75.2	377.0	394.0	84.9	240.4	196.0	464.0

DT determinate, *NDT* non-determinate

the natural hybrids of *C. sericeus* and *C. albicans*. These plants were characterized by distinct spreading plant growth (dominant trait). Similarly, the other dominant traits such as lanceolate leaf, flat pod, and round seed shapes were prominent in the natural hybrids (Table 2). Also natural hybrid plants in general had bigger leaves and more number of long primary branches.

Benefits derived from natural out-crossing

The natural out-crossing was never considered an economically important feature of *Cajanus cajan* because it always created difficulties in maintaining genetic purity of selections and released cultivars. There are several cases where the released cultivars have been contaminated due to out-crossing within a short period of 3–4 years and consequently they have lost the primary variety traits for which they were bred (Gupta et al. 1981). On the other hand, some pigeonpea breeders have effectively used the genetic variability induced by natural out-crossing for selecting high-yielding cultivars. Singh et al. (2005) while reviewing the long term pigeonpea breeding efforts concluded that out of 63 pigeonpea cultivars released in India since 1972, 30 were selected from the variation present within the landraces that was created by natural hybridization and segregation. Cultivars such as Maruti, UPAS 120 etc., selected from variability created in farmers' fields through natural out-crossing are still popular even after 30–35 years of their releases (Singh et al. 2005).

Development of a new CMS system from a natural hybrid plant

More recently pigeonpea breeders have developed a hybrid breeding technology that is based on natural out-crossing and cytoplasmic-nuclear male-sterility (Saxena et al. 2005) system and the commercial hybrids are now reaching farmers (Saxena 2009; Saxena et al. 2009). This technology needs a regular up-grading of genetic materials by breeding new sources of CMS systems and their fertility restorers. In this context, the natural out-crossing observed in the wild relatives of pigeonpea has played an important role.

The selection of a natural hybrid plant from *C. lineatus* is showing promise towards the development a new source of CMS. In 2002 during routine observations in pigeonpea breeding materials, the authors identified a natural hybrid in a population of *C. lineatus*. This plant produced a few pods and the examination of anthers revealed that its pollen were defective with only 60% fertility. To maintain and improve the expression of male-sterility, this plant was crossed with the pigeonpea line ICPL 99044 and all the F_1 plants expressed partial male-sterility, indicating the male-sterility maintaining ability of ICPL 99044. In the BC_1 F_1 generation out of 18 plants grown, five were partial male-sterile with 70 to 98% pollen sterility and a plant showing the highest pollen sterility was selected for backcrossing. In BC_2 F_1 generation out of five plants grown, only one was male-sterile with 90% pollen sterility. In BC_3 F_1 generation, a total of 170 plants were grown and of these, 47 exhibited >90% pollen sterility. The segregation pattern among BC_4 F_1 plants for pollen sterility (Fig. 2) showed that selection for pollen sterility is effective and out of 118 plants grown, 55 had high levels (92–100%) of pollen sterility. In the remaining plants pollen fertility ranged from 10 to 80%.

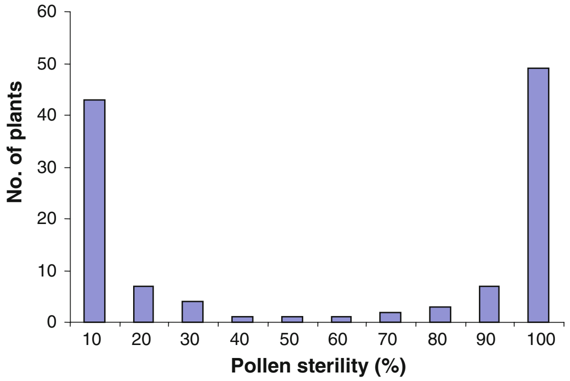

Fig. 2 Frequency distribution of pollen sterility (%) in BC_4 F_1 population

Since, the eukaryotic genome is housed not only in the nucleus but also in the cytoplasm, the nuclear genes follow Mendelian inheritance pattern while cytoplasmic genes are maternally transmitted. The interaction between the nuclear and cytoplasmic genomes results in differential phenotype due to variation in cytoplasmic contents of cells especially in early generation breeding materials. The male-fertility suppressing genes result in the expression of male-sterility due to their action that hampers the normal process of microsporogenesis. The phenotypic expression of genotypes with ‘fr’ genes is sometimes affected by complex interactions of these genes with modifiers and the macro/micro environment. The present data also showed that in BC_4 F_1 population the male-fertility (FrFr) and male-sterility (frfr) genes are slowly stabilizing as two distinct groups. This population is important from the hybrid breeding point of view since besides developing a new CMS system, the sources of its fertility restoration have also been identified. These fertile plants are likely be of immense value in studying the genetics of fertility restoration. The male-sterile BC_4 F_1 selections were used to develop BC_5 F_1 seeds for further breeding. From the hybrid pigeonpea breeding programs point of view, the development of this new CMS source is considered a major gain from natural out-crossing in wild species. This will not only enrich genetic variability among hybrid parents but also protect the crop against any potential danger of cytoplasmic uniformity, as experienced with T-cytoplasm of maize in 1970 that was responsible for diseases like southern leaf blight (caused by *Helminthosporium maydis*) and yellow leaf blight (caused by *Phyllosticta maydis*). Subsequently, the use of T-cytoplasm was virtually abandoned in hybrid maize breeding programs in the US corn belt (Kaul 1988).

Acknowledgment The authors acknowledge the help of Mr. Shrikant for typing this manuscript.

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