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Honey bee mediated (*Apis mellifera* L.) hybrid pigeonpea seed production under net house condition

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ABSTRACT

The development of cytoplasmic nuclear male sterility (CMS) hybrid technology in pigeonpea was a significant breakthrough. Hybrids produced yield 25% superior than standard varieties on farmers' field. However, the expansion and acceptance of the CMS technology depends predominantly on the efficiency of hybrid seed production. Hybrid seed is typically generated in isolated fields from other pigeonpea production areas. Finding suitable isolated fields is often difficult. Therefore, obtaining cross-pollinated seed under enclosed conditions could be a logical alternative. This was tested by using male sterile (A) and maintainer (B) lines in a 3:1 ratio under captivity (net houses) containing honeybees hives. The yield of the A line, obtained in net houses containing honeybees, was significantly lower than in open fields with natural pollinators. Intercropping pigeonpea with sunflower or spraying a sugar solution did not contribute to increase the yield of A line in the net houses. Sequential planting (weekly intervals) of A:B lines in open natural field condition was beneficial to increase the production of the A line (1078.3 kg/ha) making yields equivalent to the B line (1047.2 kg/ha). Thus, natural field isolated plots combined with sequential planting of blocks of males and females is recommended for hybrid pigeonpea seed production.

Key words: Artificial pollination, Cross-pollination, Cytoplasmic male sterility, Insects, Tur dal

Globally, pigeonpea (*Cajanus cajan* (L.) Millsp.) (also known as tur dal or gandul) is planted in around 7 m ha, mainly in tropical and sub-tropical regions of the world. India is the undisputed leader, accounting for 80% of area and 67% of the production (FAO, 2017). In India, the domestic production of pigeonpea is inferior to its demand creating a deficit of about 200,000 t per year which is met mainly with imports from Myanmar, Mozambique, Kenya Tanzania and Malawi. In spite of serious breeding efforts over decades, pigeonpea yields have been historically low with a plateau around 700 kg/ha. In this context, recent efforts to enhance productivity through hybrids have shown promise (Saxena *et al.* 2018) by increasing farm yields more than 25% over commercial varieties. Technologically, the production of seed in this three-line hybrid system is demanding and requires careful attention to preserve genetic purity of parental lines and production of bona fide hybrid seed. The female parent, a male sterile (A) line, has the cytoplasm of a wild relative of pigeonpea, *Cajanus cajanifolius,* and the nuclear genome of the cultivated type (*Cajanus cajan*) (Saxena *et al.* 2005). The maintainer (B) line is iso-nuclear to the A line but has the cytoplasm of the cultivated pigeonpea. The cross of A x B reproduces the A line. The R line, also called the fertility restorer, has both the nuclear and cytoplasm of the cultivated pigeonpea with fertility restoring nuclear gene (s). In order to generate fertile hybrid seeds (both female and male), the A line is crossed with the R line thanks to cross pollination facilitated by insects. Many crops depend on insect pollinators to successfully produce seed/fruits. Insect pollinator deficit is a very important factor in yield gap analysis (Garibaldi *et al.* 2016). Natural insect populations are subject to temporal and spatial fluctuations in quantity and type. This creates uncertainties about the final yield outcome each year. In order to overcome this situation, a number of approaches have been explored to increase the number of pollinators (i.e. installing honey beehives in farmers' fields) to attract pollinators to the crops and to increase pollinator activity (i.e. sugar solutions, pheromones, other plants with attractive flower color). In canola, seed yield was improved by 46% by installing three honey beehives per hectare (Sabbahi *et al.* 2005). Some approaches show promise and could be economically feasible. In the case of hybrid seed production using a male sterility system, there is an additional challenge, having male sterile (no pollen) lines (used as females) together with male fertile lines. In onion CMS based hybrid seed production system, male sterile lines produce less nectar in addition to having no pollen which reduces pollinators activity (Soto *et al.* 2013 and Wilkaniec *et al.* 2004). In pigeonpea, female lines also have a competitive disadvantage because they do not offer pollen to the insects and thus are likely to receive fewer visits. This is a major concern in pigeonpea hybrid seed production.

In CMS hybrid technology, the large-scale seed production of the female parent and hybrid seed involves mass transfer of pollen grains from B and R line on to A line, respectively. Since wind has no role in cross pollination in pigeonpea (Kumar and Saxena, 2001), this act is performed by insects such as *Apis mellifera, A. dorsata*, *A indica* (Pathak, 1970), *Megachile* spp. (Williams, 1977; Zeng-Hong *et al.* 2011), and *Xylocopa* spp. (Onim, 1981). The extent of natural cross-pollination varies considerably in different places (see review by Saxena *et al.* 2016); and various biological and ecological factors such as the genotypes, environment, and density of pollinating vectors determine the extent of out-crossing at a particular location.

Howard *et al.* (1919) were the first to record 2-12% out-crossing in pigeonpea in Pusa (Bihar). Other locations have reported significant level of natural cross-pollination (25-30%). Large-scale seed production of pigeonpea hybrids and their parents is typically done in physical isolations from other pigeonpea growing fields. The minimum isolation distance between pigeon fields for seed production is listed as 500 m in seed certification standards. This is always a cumbersome and expensive exercise because it is difficult to find good isolation plots for a crop like pigeonpea, which is extensively cultivated by farmers throughout India. Therefore, in order to overcome this constraint, we conducted studies to explore the possibility of producing cross-pollinated seed on the male sterile plants using honey beehives enclosed in net houses.

Our goal was also to assess if using honeybees in captivity (net houses) could be used as an effective alternative to seed production in isolated fields. Hence a few options were considered to (i) compare seed production of A x B in net houses containing honey beehives versus open conditions with natural pollinators; (ii) evaluate if intercropping with sunflower could increase seed production by helping the initial establishment of honeybees; (iii) assess if a sugar solution applied to the A plants could attractant bees and stimulate cross-pollination; and (iv) determine if weekly plantings of blocks of A x B lines could contribute to yield increases by expanding the pollen availability period.

MATERIALS AND METHODS

Plant materials: The cytoplasmic nuclear male sterile line used in the study was ICPA 2043. It contains A_4 cytoplasm from *Cajanus cajanifolius*, accession (ICPW 29), a wild relative of pigeonpea, and the nuclear genome of a cultivated pigeonpea (*Cajanus cajan*) line ICPL 20176. The B line of this male sterile (A) line is ICPB 2043. Genetically, this line is iso-nuclear to ICPA 2043 but with *Cajanus cajan* cytoplasm. When ICPA 2043 is pollinated with ICPB 2043, it produces seed equivalent to ICPA 2043. Both lines (ICPA 2043 and ICPB 2043) are of medium maturity with non-determinate growth habit. The male sterile line ICPA 2043 has been used in the development of more than 40 hybrids, being ICPH 2671 and ICPH 2740 the most promising.

Experimentation and data collection: In order to understand the processes of bee-aided cross-pollination in pigeonpea, Experiments were conducted inside net houses using honey beehives (see details about net house construction and honeybee installation below) and under natural open field conditions. The experiments were conducted in 2010 and 2011 planting multiple sets of A:B pigeonpea lines, 3:1 (3 rows of A females to 1 row of B male).

All the experiments were conducted at the research farm of ICRISAT, Patancheru, Telangana, India (17.53° N, 78.27° E, 545 ml). The experiments were planted on July 20th in 2010, and on July 7th, 15th and 21st in 2011 (sequential or staggered planting). In each experiment, sowing was done on ridges. The rows were 75 cm apart with intra-row spacing for pigeonpea seed at 30 cm. The plots (both years) were 25 m long under the net house and under open and under open pollinated field conditions. We also compiled yield data for additional seed produced under natural isolated fields at ICRISAT (Patancheru, Telangana, India) and Jabalpur (Madhya Pradesh, India)

In 2010, collected data from five sets of pigeonpea 3:1 (A:B) of each treatment: inter-cropping with sunflower, sugar spray and untreated pure crop) in a net house containing honey beehives; and five sets of 3:1 (A:B) pure crop untreated under natural open field conditions. In the tests involving sunflower inter-cropping with pigeonpea, a single row of sunflower was sown in between the sets of pigeonpea rows. A sunflower hybrid with slightly earlier flowering time than the pigeonpea lines was used. The sowing of both pigeonpea and sunflower was done on the same day. In the case of sunflower, the plant to plant spacing was 20 cm. The idea behind using the inter-planting of sunflower was to assess if inter-cropping would attract/ assist more pollinators and thus contribute to increase cross-pollinated A x B seed yield. Similarly, the tests involving the spray of a sugar solution on the A lines were intended to increase bee attraction. This operation (spraying sugar solution) had been used by farmers in some parts of India to attract pollinators. If found effective, it could be used to increase productivity of cross-pollinated seed for maintaining the male sterile A line (A x B) and also to produce hybrid seed (A x R). A 10% sugar solution was sprayed on 25th October and 9th November between 13:00 to 14:00 h to the treated set (5 reps of 3:1 A:B) whereas the other set was left untreated.

In 2011, we conducted experiments in a net house (with honey beehives) and under natural open conditions to figure out if the extend of flowering duration through staggered planting of A and B lines would improve pod set on the male sterile plants (2011). The male and female lines were sown on three times, July 7, 15, and 21, with approximately one week in between. We planted a central block of eight sets of 3A:1B followed by four sets of 3A:1B a week later on both sides (four sets on the right and four sets on the left) and another planting of two weeks later also on both sides.

Standard agronomic practices were followed to raise a normal crop in both the years. These included basal irrigation to ensure good moisture for germination, application of 100 kg ha^{-1} of Di-ammonium phosphate, pre-emergence herbicide application of Stomp and Paraquat @ 2 and 4 L ha^{-1}, respectively. In addition, one hand weeding was also done at early vegetative stage. Irrigation was provided when necessary to ensure regular emergence of new flowers. To control the pod damage by *Helicoverpa armigera,* a single insecticide (Spinosad @ 0.2 L ha^{-1}) spray was done one week before installing the honeybeehives during 2010, and twice in the 2011 experiment after 6 p.m. so that the pollinators were not affected.

Yield and flowering data were recorded on a per plot basis. In order to collect data on seeds/pod, seed yield per plant (g) and 100-seed weight (g), 10 randomly selected individual plants per row were used.

Construction of net houses: The net houses for the experiments were constructed using nylon black mosquito nets. The net houses were 3 m in height and covered an area of 75 m x 25 m in both 2010 and 2011. To support the nets, 3 m tall iron poles were attached to the ground in the corners and middle of the net. For providing lateral support to net roofing, light-weight aluminum frames were used to reinforce support provided by iron poles. For entry into the net house, a section with two mesh doors was erected and attached to the main net house to control the migration of bees.

Bee management: The honeybee (*Apis mellifera* L.) frames were obtained by Mr. Noorbasha Kaleshavali from Andhra Pradesh (India) and multiplied by the ICRISAT Integrated Pest Management team. To acclimatize honeybees to net house environment, the bees were fed with 50% sugar solution for the first couple of days. When pigeonpea was at its peak flowering stage, beehives were installed in the middle of the net house to improve the bee population and one brood frame was placed in the hives every week to compensate the mortality of foraging bees. The 2010 trial was partitioned into two portions (intercropping side, and pure crop side) and each part was provided with two seven-framed beehives with each frame housing approximately 2000 bees. In 2011, the number of beehives was increased to four with 7 frames each. The beehives were placed in the center of he netted area. As the bees having natural tendency to move towards east, the hives were placed on a wooden stool at two feet height with entrance facing east. To provide fresh water to the bees, water was filled daily to the four metal trays placed below the stool and one in front of the hives. To maintain the supply of healthy brood frames to the experimental area several beehives were maintained on the farm. The beehives were removed after cessation of flowering, the first week of december.

The results were analyzed with the statistical package JMP (JMP Pro 13, 2016) using the Standard Least Square personality and the Effect Leverage emphasis option. All factors were considered fixed. The means of the A lines were compared with the means of the B lines within and between treatments under different scenarios: Pure pigeonpea crop 3:1 (A:B lines) under open field conditions with natural pollinators; pure pigeonpea crop 3:1 (A:B) in net house with honeybees; sunflower intercropping in net house with honeybees, sugar spray in net house with honeybees; and sequential planting in open pollinated field versus in net house with honeybees. For the mean comparison, Least Square means were generated and compared using L S Means Student's t. A significance level of $P<0.05$ was used.

RESULTS AND DISCUSSION

Effect of net houses and honeybees on seed yield of the pigeonpea male sterile line: Pigeonpea flowers are prone to cross-pollination because they are not truly cleistogamous and the nectar produced by flowers attracts bees. Nectar production in flowers is regulated by a phyto-hormone called jasmonic acid that is produced in floral nectaries endogenously (Radhika *et al.* 2010). Kumar *et al.* (2009) observed that the nectar production in pigeonpea flowers remains consistent throughout the day across the entire flowering duration. Besides nectar production, there are factors such as extended stigma receptivity (Dalvi and Saxena, 2009) and competitive advantages of foreign pigeonpea pollen (Mazi *et al.* 2014) which also encourage cross-pollination in pigeonpea. Foreign pollen germinates faster (Reddy and Mishra, 1981) and the pollen tube grows at a faster pace than the self-pollen to affect cross-fertilization (Dutta and Deb, 1970).

For foraging, the pollinator bees extend their proboscis to rob the nectar and during this process they brush the anthers and a load of pollen gets stuck on various body parts such as hairs, silk, legs, mouthparts, thorax, and abdomen. Williams (1977) estimated that each pollinating insect carried a load of about 5,000 to 90,000 pollen grains on its body. The cross-pollination occurs when the pollen-laden insects trip onto other flowers and rub their bodies on stigmatic surface. However, with respect to foraging period on pigeonpea flowers the reports are quite variable. Onim (1981) recorded that each insect visit to pigeonpea flower lasted for 15-55 seconds; while Pando *et al.* 2011 recorded a high foraging speed of 10.3 flowers/ min. Zeng-Hong *et al.* (2011) reported that the pollinating insects, on average, visited 4.8 flowers/10 minutes. Mazi *et al.* (2014) reported that bees, on average, sat on a flower for 28 seconds to collect pollen, 43 seconds to collect nectar, and for 63 seconds to collect both nectar and pollen.

Using net houses to produce seed of the male sterile A line (ICPA 2043 x ICPB 2043) ensured isolation. The mosquito net did not allow insects (carrying pollen from other fields) to come inside the net house. Honey beehives inside the nets provided pollinators to move pollen between plants. The effect of using a net house containing honeybees versus natural open pollination was compared (Table 1). Flowering took place earlier in the net house than in the open field, but no significant differences were observed between the A and B lines within each treatment. The A line had larger seeds (both under net and open field) but it was more evident in the net house, likely explained by enhanced mobilization of nutrients to a small number of seeds (Table 1). Open field conditions with natural insect pollinators were especially beneficial to increase the yield of the A line (789.8 kg/ha), but honeybees inside the net house did not generate the anticipated benefits (the A line only produced 296.5 kg/ha in the net house). The total yield of the A line under natural field conditions was almost three times higher than inside the net house (Table 1). The yield of the B line under natural field conditions (1,300.8 kg/ha) was significantly higher than both the A and B line in the net house with honeybees (Table 1) and also A line under natural open field conditions. Despite being a promising idea, planting pigeonpea A:B lines in net houses with honeybees is not recommended to produce seed of the male sterile A line because the yield of the A line was very low. According to the information published by Savoor (1998), the use of beehives started in 1892, for increasing the efficiency of pollination and thereby enhancing crop yields, and by 1940 the pollination services were commercialized in USA. These services are frequently used glasshouse-grown tomatoes (Cribb *et al.* 1993). In *Brassica napus* the hybrid program suffers due to ineffective pollination. It happens because the pollinator bees (*A. mellifera*) confine on the fertile plants and seldom visit the male sterile plants. Rajkhowa and Deka (2016) studied the effect of bee (*A. cerana*) population on pigeonpea pod set and yield and reported that by installing 5 beehives/ha in an open field, the pod set enhanced by 78% with a yield advantage of 138% over the control treatment *i.e.* open field crop without bee hive with cost benefit ratio of 1:1.49.

Effect of sunflower intercrop on seed yield of the pigeonpea male sterile line: Inter-planting pigeonpea lines with a sunflower cultivar was aimed to facilitate the initial establishment of the bee population and to increase bee movement over pigeonpea plants to enhance pod set on the pigeonpea male sterile plants. Sunflower plants flowered two days earlier than the pigeonpea lines ICPA 2043 and ICPB 2043 (data not shown). Inside the net houses, both pigeonpea lines (A and B) took similar time (about 98 days) to flower. Seed yield of the B line (on a per plant and per plot basis) was significantly higher than the yield of the A line under both conditions, sole crop and inter-cropped with sunflower (Table 2). The effect of inter crop was not visible on the productivity on either of the lines. The male sterile lines possessed, in general, larger seed than their maintainer (B) counterparts; this could be due to enhanced nutrient mobilization and allocation to a reduced number of seeds. Ours observations showed the inter-planting sunflower with pigeonpea could not attract more bees to pollinate pigeonpea to influence pod set on the male sterile plants. Thus, this practice is not recommended. The results of the experiments we conducted in net houses using honeybees indicated that on average, the yield produced by the B line was much higher than the yield produced by the A lines: 69.0% advantage in 2010 and 86.3% advantage in 2011 (sequential planting). Under open field conditions, the yield of the B line was superior to that of the A line in 2010 (39.2% superiority), but the yields of the A and B lines were equivalent under field conditions when sequential planting was used (2011) (no superiority of the B line). Our data also shows that the imposition of net a net house and honeybees to the A line, resulted in yield losses instead of increases (62.5% yield loss in 2010 and 84.6% in 2011 with

Table 1. Effect of using net houses containing honeybeehives on flowering and yield of ICPA 2043 and ICPB 2043 (2010, ICRISAT, Telangana, India)

Treatment	A/B lines	Days to flower	100-Seed weight (g)	Yield/ plant (g)	Grain yield (kg/ha)
Under net†	ICPA 2043	97.4 b	13.2 a	6.7 c	296.5 c
	ICPB 2043	97.5 b	10.4 d	21.5 b	956.8 b
Mean under net		97.4 B	11.8 A	14.1 B	626.7 B
Open field	ICPA 2043	104.0 a	11.5 b	17.8 b	789.8 b
	ICPB 2043	102.9 a	11.1 c	29.3 a	1300.8 a
Mean open field		103.4 A	11.3 B	23.5 A	1045.3 A

† Pure crop, no sugar spray.

Values connected with the same letter within font type are not significantly different according to Student's t test at probability 0.05.

Table 2. Effect of inter-cropping pigeonpea with sunflower on flowering and yield of ICPA 2043 and ICPB 2043 in net houses containing honey beehives (2010, ICRISAT, Telangana, India)

Treatment	A/B lines	Days to flower		100-Seed weight (g)		Yield/ plant (g)		Grain yield (kg/ha)	
Pure crop	ICPA 2043	97.4	a	13.2	a	6.7	b	296.5	b
	ICPB 2043	97.5	a	10.4	b	21.5	a	956.8	a
Mean pure crop		97.4	A	11.8	A	14.1	A	626.7	A
Inter crop	ICPA 2043	97.4	a	13.2	a	4.9	b	219.4	b
	ICPB 2043	98.0	a	10.5	b	23.9	a	1060.6	a
Mean inter crop		97.7	A	11.8	A	14.4	A	640.0	A

Values connected with the same letter within font type are not significantly different (according to Student's t test at probability < 0.05).

sequential planting). The yield of the B line was significantly higher in open fields than under net conditions in 2010, but the advantage was only 26.4% (1,300 vs 956.8 kg/ha) (Table 5). In 2011 (sequential planting) the yield of the B line was superior in net houses (Table 5). Based on the plot yield data, the B line produced more than a ton per hectare yield under both net houses and open field (Table 5). Seed production data of A line in isolations fields at Jabalpur (781 kg/ha) and Patancheru (875 kg/ha) were comparable with that of open field mean yield (789.8 kg/ha in 2010) using similar planting scheme (3:1 A:B and single planting). Based on this study, we would not recommend using *A. mellifera* in captivity to cross- pollinate the male sterile flowers of pigeonpea since the yields would be low. Producing seed in isolation fields is preferred. If isolation fields are an issue, it is possible to produce B line (or commercial varieties) in captivity using net house and honeybees in combination with sequential planting since the resulting yields would be equivalent or slightly higher (15%) than using natural open field conditions. A careful evaluation of the pros and cons of open fields versus cages with honeybees should be considered. In any case, production of seed in cages with honeybees is not recommended for the production of the sterile A line (A x B) nor for the hybrid (A x R).

Effect of spraying sugar solution on seed yield of the pigeonpea male sterile line: According to Jay (1986) and Currie (1997) the spraying of specific substances on the target crops can potentially enhance cross-pollination by attracting insect pollinators. Spraying sugar solution was considered as an option to attract honeybees and thus increase seed production. Under captive conditions, the spraying of sugar solution on parental lines ICPA 2043 and ICPB 2043 resulted in a delay in flowering (two days) but flowering time was not significantly different when comparing the A and B lines within each treatment. There was also a reduction of plant yield when applying the sugar solution, however it was not enough to declare significant differences. The yield reduction was more noticeable in the case of the male sterile plants (ICPA 2043). In both situations (sprayed and unsprayed) ICPA 2043 produced lower yields than ICPB 2043. ICPB 2043 had larger seeds independently of the sugar treatment (Table 3). Our results indicated that spraying a sugar solution did not significantly increase pigeonpea seed production on the A line as initially anticipated (Table 2, Table 3). By contrast, Currie *et al.* (1992) and Winston and Siessor (1993) reported that spraying of synthetic pheromones increase honeybee foraging activity and crop yield under a wide range of conditions. Margalith *et al.* 1984 reported that spraying of Beeline a food supplement, enhanced significantly the bee activity as well as cross-pollination in cucurbits. Sagili *et al.* 2015 used honeybee brood hormone in hybrid carrot production and reported around 18% yield gain. Variation in nectar sugar concentration had little direct bearing on bee activity on different fruit crops (Abrol, 1993).

***Effect of staggered plantings on seed yield of the pigeonpea male sterile line*:** To assess the effects of different planting times on the efficiency of bees in cross-pollination, the set of A:B lines were sown on three dates each separated by seven days. There were some differences in flowering time between planting dates, but they were consider minor. In all cases, the A line flowered a little later (two to three days). Seed weight in the net house (2011 sequential planting experiment) followed a similar pattern than that observed in 2010 (both in the net house and open field): The A line had significantly larger seed than the B line. However, seed weight was similar between the A and B lines (around 9.5 g) under natural field conditions when sequential planting was used. Under net conditions, the A line had less seeds per pod than the B line. However, the number of seeds per pod was similar between the A and B lines under natural field conditions when sequential planting was used. Seeds per pod were not evaluated in 2010, but visual observations indicated that the A lines had less seeds per pod than the B line in all cases (open field, intercropping and sugar solution treatment). The yields of A line under captive environment in the three sowing dates ranged from 3.5 to 4.2 g/plant; while under the same net the B lines produced from 26.0 to 30.8 g/plant. The ratio of B/A for plot yield was 6.1, 7.0 and 8.9 for dates 1, 2 and 3, respectively (inside net). The B line reached the maximum yield (1,368.9 kg/ha) when planted in date 3 (21 July) but the yield of the A line was not affected by planting date in the net house (Table 4). Under open field conditions, the yield data of the A and B lines was not significantly different within each planting date. The productivity of B line was similar to the A line (almost 1:1) (Table 4). The first planting date resulted in the highest yield for both A and B line (1,200.8 and 1,212.6 kg/ha, respectively), probably

Table 3. Effect of spraying sugar solution on flowering and yield of ICPA 2043 and ICPB 2043 in net houses containing honeybeehives (2010, ICRISAT, Telangana, India)

Treatment	A/B lines	Days to flower	100-Seed weight (g)	Yield/ plant (g)	Grain yield (kg/ha)
No spray	ICPA 2043	97.4 a	13.2 a	6.7 b	296.5 b
	ICPB 2043	97.5 a	10.4 b	21.5 a	956.8 a
Mean no spray		97.4 B	11.8 A	14.1 A	626.7 A
Sugar spray	ICPA 2043	99.1 a	13.0 a	3.6 b	161.0 b
	ICPB 2043	99.4 a	10.8 b	19.9 a	883.7 a
Mean sugar spray		99.3 A	11.9 A	11.8 A	522.3 A

Values connected with the same letter within font type are not significantly different (according to Student's t test at probability < 0.05).

benefiting from exposure to extended pollen availability in the environment (Table 4). Stagger planting (weekly plantings for three weeks) under open field conditions with natural insects contributed to increase the yield of the A line, but sequential planting in the net house (with honeybees) did not. Sequential planting in the net house containing honey beehives was effective, nevertheless, to produce good yield of the B line (above 1,200 kg/ha).

In summary, based on the results of this study it is clear that the placement of honey beehives inside the net house environments, intended to increase seed yield of male sterile (A x B) lines and hybrid (A x R), is not recommended. Honeybees were less attracted by male sterile lines, probably due to the lack of pollen, which resulted in low yields. Intercropping with sunflower and spraying sugar solution did not help increase yield of A lines. Sequential planting could be recommended but it does not solve the need for isolation fields. We believe that sequential planting increased the pollen availability period and thus benefited the three planting dates since pigeonpea flowers were available to be pollinated for an extended period of time. This, combined with the fact that natural insects were active and ready to pollinate made the A x B production a success. The relative yield comparison of A vs B in 2010 versus 2011 suggests that staggering was beneficial, to the point of obtaining yield of A lines was equivalent to the yield of the B lines. Higher yields could be explained by the fact that pollen was available for an extended period and to higher pollinator diversity under natural open field conditions. Thus, staggered planting is a recommended option to increase yield obtained by cross-pollination under natural field conditions. This would need to take place in isolated fields with presence of natural pollinators. There may be a possibility that, the natural pollinators (*Apis cerena*, *Apis dorsata*) in open field conditions are more active and efficient in pollination compared to the introduced *Apis mellifera* in pigeonpea. Exploring the native bee species under captive conditions is not possible due to their aggressive nature. Under the captive conditions, the honeybees are observed to be panicked and mostly found trying to escape the caged condition. More efforts should be explored to make the honeybees more comfortable with the captive conditions so that most of the energy invested in foraging ultimately increasing the seed yield. Further, it is advisable to explore the use of other natural pollinating insects (Greenleaf and Kremen, 2006; Pitts-Singer and Cane, 2011). Among then, *Megachile* species would be a good candidates or trying the native species such as *A. indica,* which has a short distance foraging habit, for hybrid seed production in pigeonpea because they frequently visit pigeonpea flowers and, thus they should also be explored for the production of cross-pollinated seeds on the male sterile plants under captive conditions. In conclusion, we recommend the use of staggered/sequential weekly plantings in isolated field for pigeonpea hybrid seed production, both A x B (production of the female A line) and A x R (hybrid seed). Production of clean pigeonpea seed using net houses and honeybees, combined with sequential planting, is a feasible alternative to produce male fertile B lines or commercial varieties if isolation fields are

Table 4. Effect of three planting dates on flowering and yield of the male sterile line ICPA 2043 and its maintainer ICPB 2043 line grown inside net houses with honeybeehives and in open field conditions with natural insects (2011, ICRISAT, Telangana, India)

	Date of Sowing	A/B lines	Days to flower		100-Seed weight(g)		Seeds/ pod		Yield/ plant (g)		Grain yield (kg/ha)	
Under net	Date 1 (July 7)	ICPA 2043	104.9	a	10.3	b	1.9	b	4.2	e	186.7	e
		ICPB 2043	101.3	e	9.5	cd	3.3	a	26.0	bc	1155.2	bc
		Mean date 1	103.1	CD	9.9	B	2.6	B	15.1	DE	671.0	DE
	Date 2 (July 15)	ICPA 2043	105.0	a	11.4	a	2.0	b	3.6	e	157.8	e
		ICPB 2043	102.5	cd	9.8	bcd	3.3	a	24.9	bc	1108.4	bc
		Mean date 2	103.8	AB	10.6	A	2.6	B	14.2	E	633.1	E
	Date 3 (July 21)	ICPA 2043	105.0	a	11.7	a	2.0	b	3.5	e	154.0	e
		ICPB 2043	103.0	c	9.9	bc	3.3	a	30.8	a	1368.9	a
		Mean date 3	104.0	A	10.8	A	2.7	B	17.1	D	761.5	D
		Mean ICPA 2043	105.0	A	11.1	A	2.0	C	4.2	C	166.2	C
		Mean ICPB 2043	102.3	C	9.8	B	3.3	B	27.2	A	1210.8	A
Open field												
	Date 1 (July 7)	ICPA 2043	103.2	c	9.5	cd	3.4	a	27.3	b	1212.6	b
		ICPB 2043	101.9	de	9.7	cd	3.6	a	27.0	ab	1200.8	ab
		Mean date 1	102.5	E	9.6	BC	3.5	B	27.2	A	1206.7	A
	Date 2 (July 15)	ICPA 2043	104.6	a	9.5	cd	3.6	a	22.6	cd	1003.8	cd
		ICPB 2043	102.0	de	9.3	cd	3.5	a	19.3	d	857.2	d
		Mean date 2	103.3	BC	9.4	C	3.5	B	21.0	C	930.5	C
	Date 3 (July 21)	ICPA 2043	103.9	b	9.4	cd	3.4	a	22.9	c	1018.5	c
		ICPB 2043	101.3	e	9.2	d	3.6	a	24.4	bc	1083.6	bc
		Mean date 3	102.6	DE	9.3	C	3.5	B	23.7	B	1051.0	B
		Mean ICPA 2043	103.9	B	9.5	B	3.5	AB	24.3	B	1078.3	B
		Mean ICPB 2043	101.7	D	9.4	B	3.6	A	23.6	B	1047.2	B

.Values connected with the same letter within font type are not significantly different (according to Student's t test at probability < 0.05).

Table 5. Summary of mean yields of A and B lines recorded at ICRISAT (Telangana, India) in captivity (net houses with honeybees) and on open fields in isolation at Jabalpur (Madhya Pradesh, India) and Patancheru (Telangana, India)

		Plant yield (g/plant)		Plot yield (kg/ha)		
Location	**Genotype**	**Open field**	**Under net**	**Open field**	**Under net**	**% loss**
ICRISAT	Sterile (A line)	17.8	6.7	789.8	296.5	62.5
	Fertile (B line)	29.3	21.5	1300.0	956.8	26.4
	% Diff. (B vs A)			39.2	69.0	-
ICRISAT†						
	Sterile (A line)	24.3	4.2	1078.3	166.2	84.6
	Fertile (B line)	23.6	27.2	1047.2	1210.8	-15.6
	% Diff. (B vs A)			-3.0	86.3	-
Jabalpur‡	Sterile (A–line)			781.0		
Patancheru‡	Sterile (A–line)			875.0		

†Sequential planting (July 7, July 15, July 21) ‡Isolation seed production plots

not available but not for A line production (A x B) nor for hybrid seed production (A x R).

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