Progress in interspecific hybridization between *Cicer arietinum* and wild species *C. bijugum*

Nalini Mallikarjuna[1]*, Deepak Jadhav, V Nagamani, C Amudhavalli and **DA Hoisington**

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324, Andhra Pradesh, India

*Corresponding author: n.mallikarjuna@cgiar.org

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Cultivated chickpea (*Cicer arietinum*) rests on a narrow genetic base because of its single domestication event (Zohary 1996) and very high self-pollination rate of 98–100% (Gowda 1981). Adequate sources of resistance to various biotic and abiotic constraints are often not available within the cultivated germplasm and this has stimulated the interest to use wild species for the improvement of chickpea. Wild species are not only good sources of resistance to various biotic and abiotic constraints, but contribute genetic variation for the construction of genetic linkage maps (Winter et al. 2000) for accelerated breeding.

There are 43 wild species of *Cicer,* 8 of which are annual and the remainder perennial. Except for two annual *Cicer* species, *C. reticulatum* and *C. echinospermum*, none of the annual or perennial *Cicer* species have been successfully crossed with cultivated chickpea using conventional hybridization techniques, and hybrids obtained (Stamigna et al. 2000). Amongst the annual wild *Cicer* species there is interest to use *C. bijugum* for the improvement of cultivated chickpea as *C. bijugum* has multiple disease resistance (Robertson et al. 1995). There is no report of successfully crossing *C. bijugum* with cultivated chickpea and producing hybrids. In this investigation, we report the production of hybrid plants between *C. arietinum* and *C. bijugum* in vitro and the transfer of hybrid plants to soil (Fig. 1).

Four chickpea cultivars, ICCV 92318, ICCV 2, ICCV 10 and KAK 2, were used as female parents. The wild species *C. bijugum* (accession ILWC 73) from germplasm collection at the International Center for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria was used as male parent. Seeds of cultivated and wild chickpea were sown in the field as well as in the glasshouse. Crosses were carried out from October 2001 to February 2005 (four postrainy seasons) in the field. Flower buds were emasculated and tagged in the afternoon (15:00 to 16:00 h). They were pollinated with fresh pollen of ILWC 73 the following morning (before 10:00 h). Pods from self-pollinations on the same branch were removed to facilitate the formation of pods from cross-pollinations. Application of a combination of growth regulators consisting of gibberellic acid (50 mg L^{-1}), naphthaleneacetic acid (10 mg L^{-1}) and kinetin (10 mg L^{-1}) to the base of pollinating pistils postponed the abscission/abortion of pods from 3–6 days to 18–22 days.

Pods, which began to turn yellow, were harvested, dissected and the ovules/immature seeds with aborting embryos were cultured on the ovule culture medium. This medium consisted of B5 basal salts supplemented with 0.25 mg L^{-1} indole-acetic acid and 1 mg L^{-1} zeatin. Ovules more than 2.5 mm in length were rescued by the in-ovulo embryo rescue technique standardized for chickpea wide crosses (Mallikarjuna 1999). Embryos emerged out of the ovules 3–4 weeks after ovule culture. Hybrid seedlings were cultured on embryo growth medium consisting of ML-6 basal medium (Kumar et al. 1988) supplemented with 2 mg L^{-1} benzylaminopurine and 0.5 mg L^{-1} indole-acetic acid.

Some of the hybrid seeds of *C. arietinum* and *C. bijugum* crosses germinated in vitro giving rise to a well developed root and shoot system. It was possible to transfer such seedlings to soil directly instead of grafting the shoots to chickpea stocks (Mallikarjuna et al. 2005). Robust shoots were rooted in vitro by initially treating the shoots with 100 mg L^{-1} of indole-butyric acid for 5 days and transferring the shoots to ML-6 basal medium. Well-developed roots appeared at the cut end in 18 to 20 days after treatment.

DNA was extracted from young leaflets using Qiagen miniprep kits according to manufacturer's protocols (Qiagen, Valencia, CA, USA). For SSR (simple sequence repeat) analysis, PCRs (polymerase chain reactions) contained 50 ng of genomic DNA, 2 µm each of forward and reverse primer, 1.5 mM magnesium chloride, 200 µM DNTPs, 1 unit of taq DNA with 1x reaction buffer in a total reaction volume of 20 µl. Reaction conditions were 96° C for 2 min, 35 cycles of 94°C for 20 sec, empirically defined annealing temperature of 55°C for 50 sec and elongation at 60°C for 50 sec. Amplification products

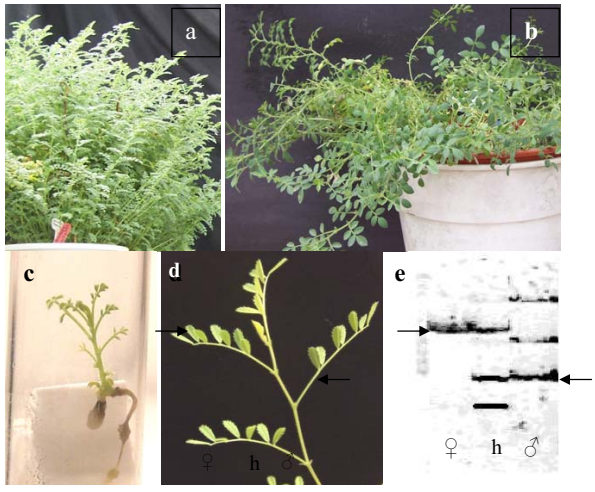

Figure 1. Interspecific hybridization between *Cicer arietinum* and *C. bijugum*: (a) Cultivated chickpea *C. arietinum*; (b) Wild species *C. bijugum*; (c) In-ovulo embryo rescue; (d) A portion of hybrid twig enlarged to show its hybrid morphology; (e) SSR analysis of the parents and hybrid. Arrows point at the bands contributed by each parent, present in the hybrid (h).

were visualized on non-denaturing 9% [29:1 (w/w)] polyacrylamide gel followed by silver staining. Silver staining consisted of 3 min in water, 20 min in 0.1% (w/v) CTAB, 15 min in 0.3% ammonium solution, 15 min in a solution of 1M sodium hydroxide, 0.1% silver nitrate and a few drops of 25% ammonium solution, and a rinse in water, followed by development in a 1.5% sodium chloride solution with 0.02% by volume formaldehyde solution.

Pod set was obtained only after the application of growth regulators to the pollinated pistils. Pods did not set when cross-pollinations were not followed by hormone applications. Pod formation varied from 18 to 65% in the four crosses (Table 1). Pod formation was highest (65%) in the cross ICCV 2 × ILWC 73 (Table 1). In the cross ICCV 2 × ILWC 73, a large number of pods were devoid of seeds inside or the seeds were small (<2 mm). Twenty-two ovules from the cross were cultured, of which 32% germinated (Table 1). Pod formation was 38% in the cross KAK 2 × ILWC 73. A total of 13 ovules were cultured from this cross (Fig. 1c), with 3 ovules germinating and all three gave rise to a well-developed shoot and root system. The seedlings were directly transferred to soil. The hybrid had a trailing growth habit as observed in *C. bijugum*; flowers were fragile and pale violet in color, and were very few in number.

In the other crosses, germinating embryos were transferred to fresh ovule growth medium without disturbing the seed coat, which in most of the seeds was sticking to the growing embryos. The root system of the hybrid plants was not robust, being fragile and at times the older roots turned brown, not comparable to the root system of the cultivated or wild chickpea. Two seedlings from the cross ICCV 2 × ILWC 73 and three seedlings from the cross KAK 2 × ILWC 73 did have a robust root system. Seedlings from the cross ICCV 2 × ILWC 73 grew for 6 weeks and later necrosed. Two seedlings from the cross KAK 2 × ILWC 73, although slow in growth, survived the transfer to soil. Hybrid plants had a trailing growth habit comparable to the wild species, the pollen parent *C. bijugum*. Morphology of the leaves was intermediate between the two parents, but largely resembling *C. bijugum* (Fig. 1a, b and d). SSR marker GA26 distinguished the parents from each other. The hybrid had one unique band from each parent (Fig. 1e) thus confirming its hybridity.

Pollination can induce pod formation in chickpea wide crosses, but for hybrid seed set, the application of growth regulators was mandatory in cross-pollinations

Table 1. Results of the crosses between *Cicer arietinum* and wild species *C. bijugum*.

Cross	No. of pollinations	No. of pods	Percentage of pods formed	No. of ovules cultured	No. of ovules germinated
ICCV 2 × ILWC 73	202	132	65	22	7
KAK 2 × ILWC 73	551	210	38	13	3
ICCV 92318 × ILWC 73	133	31	23	9	3
ICCV 10 × ILWC 73	179	33	18	6	1

using *C. bijugum*. The genotype of the female parent had a major role to play in pod set from cross-pollinations. Cultivars KAK 2 and ICCV 2 were found to be better female parents than ICCV 10 and ICCV 92318.

Since mature hybrid seeds were not obtained, the barrier to hybridization is post-zygotic as reported by Mallikarjuna (1999), Stamigna et al. (2000) and McNeil et al. (2007). The in-ovulo embryo rescue technique developed for *C. arietinum* × *C. pinnatifidum* (Mallikarjuna 1999) was effective for the cross *C. arietinum* × *C. bijugum*. The hybrids were able to synthesize their own chlorophyll; hence addition of zeatin to the medium used to water the hybrid plants during growth, which was essential for the crosses with *C. pinnatifidum* (Mallikarjuna et al. 2005), was not required

Formation of viable green hybrid plants from the cross *C. arietinum* and *C. bijugum* with intermediate morphology between the two parents show that it is possible to obtain hybrid plants between *C. arietinum* and *C. bijugum* and that concerted efforts will yield hybrids in large numbers. Our study confirms that it is possible to cross *C. bijugum* with cultivated chickpea, and it would be feasible to produce a large number of hybrids to exploit the genes/traits present in *C. bijugum* for the improvement of cultivated species.

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