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Ovule and embryo culture to obtain hybrids from interspecific incompatible pollinations in chickpea

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Summary

Many of the wild species of chickpea were not accessible to the improvement of chickpea due to cross incompatibility. In these interspecific incompatible crosses, fertilization takes place but the embryo aborts a few days later. The only way to obtain hybrid plants is by the application of growth regulators to pollinated pistils to prevent initial pod abscission and to save the aborting hybrid embryos by embryo rescue techniques. Although there are a few papers on regeneration from different explants of chickpea, information on embryo rescue techniques is not available. The paper summarises the embryo rescue techniques developed for chickpea, by the use of which hybrid plants between *C. arietinum* and *C. pinnatifidum* were produced. The paper also emphasises the effect of genotype to successfully obtain hybrids. The morphology of the hybrid plants resembled the male parent in leaf structure and growth habit. The colour of the flowers produced on the hybrid plant was pale violet, resembling the male parent whose flowers were violet in colour. The flower colour of the female parent was white.

Introduction

The genetic diversity in wild relatives of chickpea have been little utilized for the improvement of cultivated chickpea. The genus *Cicer* comprises of nine annual species, of which *C. arietinum* is cultivated. Of the eight wild species only *C. reticulatum* and *C. echinospermum* have been successfully crossed with chickpea (Ladizinsky & Alder, 1976; Pundir & Mengesha, 1995). Crosses with other wild species have not been successful. Apart from the annual wild *Cicer* species, there are 33 perennial wild species which have not been successfully crossed with cultivated chickpea.

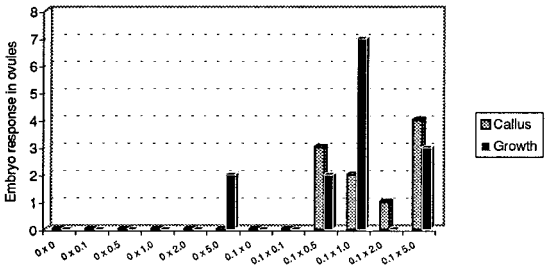
Wild species of chickpea are particularly interesting because they are useful sources of genes for tolerance to abiotic and biotic stresses (Singh et al., 1997) and a good source of resistance to ascochyta blight (Smithson et al., 1985) and the only source of resistance to botrytis grey mold (Haware et al., 1990).

There is ample evidence that the barriers to hybridization between in-compatible wild species of *Cicer* and cultivated chickpea are post-zygotic (Ahmad et al., 1988). This is exemplified by the fact that the pollen grains germinate on the stigma and fertilization takes place. The embryos abort soon after. In such instances the only method by which hybrids can be obtained, is by saving the hybrid embryos through embryo rescue techniques.

Although regeneration techniques are now available for chickpea, the techniques to save aborting embryos from interspecific in-compatible crosses are not. The present communication describes the embryo rescue techniques developed for chickpea, by the use of which hybrid plants between *C. arietinum* and *C. pinnatifidum*, an interspecific incompatible wild species of chickpea, were obtained.

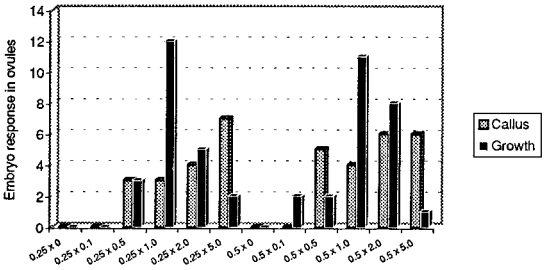
Material and methods

The ovule (immature seed) culture was standardised with selfed ovules of chickpea variety ICCV 10, because the large number of ovules needed for exper-



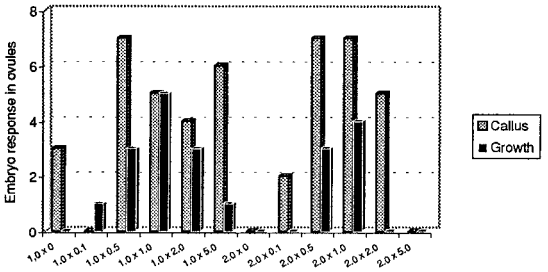
Callus	0	0	0	0	0	0	0	0	3	2	1	4
Growth	0	0	0	0	0	2	0	0	2	7	0	3

IAA x ZEATIN (mg/lt)



Callus	0	0	3	3	4	7	0	0	5	4	6	6
Growth	0	0	3	12	5	2	0	2	2	11	8	1

IAA x ZEATIN (mg/lt)



Callus	0	0	3	3	4	7	0	0	5	4	6	6
Growth	0	0	3	12	5	2	0	2	2	11	8	1

Figure 1. Embryo growth on different combinations of IAA and Zn in selfed ovules, 10 days after pollination.

imentation could not be obtained from interspecific pollinations.

The flowers were tagged on the day of opening and harvested at 6, 8, 10 and 12 days after pollination (DAP). Ovules were dissected out of immature pods and cultured.

ML-6 medium (Kumar et al., 1988) was used as the basal medium for ovule culture at pH 5.6 with 3% sucrose. Indole-3-aceticacid (IAA) at 0.0, 0.1, 0.25, 0.5, 1.0 and 2.0 mg L-1 and Zeatin (ZN) at 0.0, 0.1, 0.5, 1.0, 2.0 and 5.0 mg L-1 were used in different combinations. The liquid medium was autoclaved in stock quantities and later dispensed into aseptic vials containing filter paper bridges. Three ovules were cultured in each vial and there were 6 vials per hormone combination. Ovules were incubated at 26 °C under 18 hrs light and 6 hrs dark regime. For light microscopic observation of embryo growth, ovules were fixed in 70% alcohol after 30 days of culture.

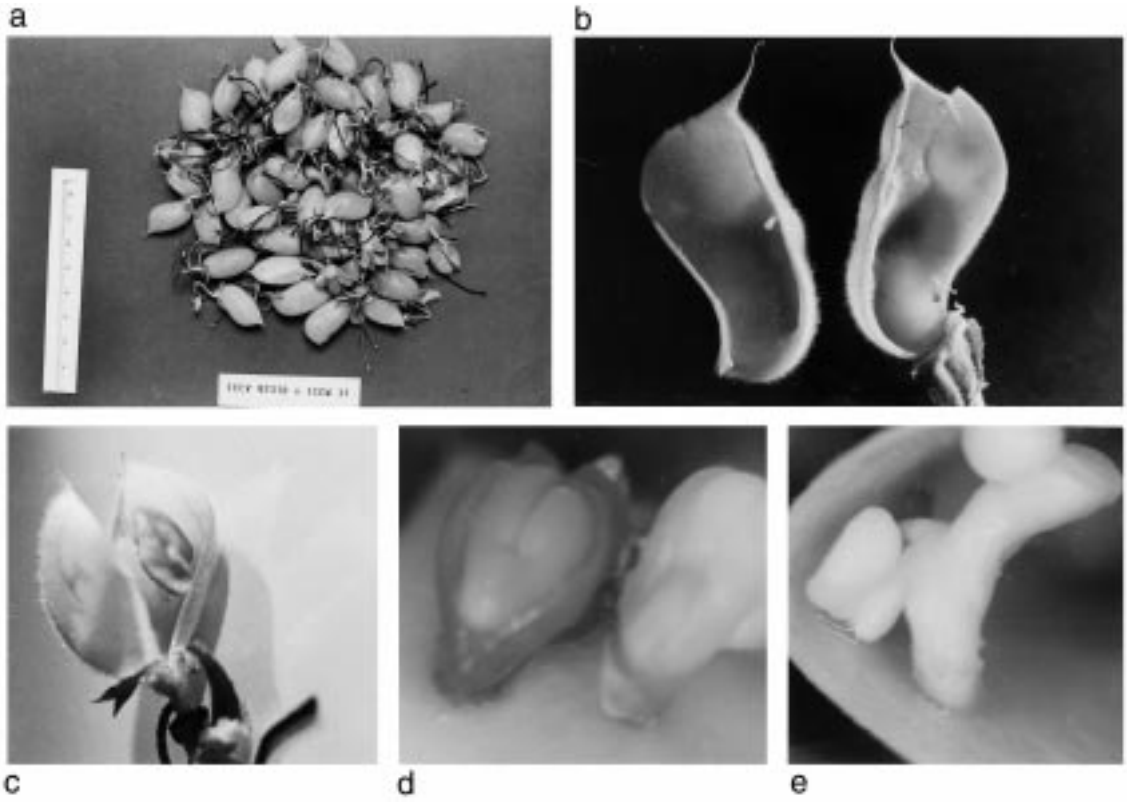


Figure 2. Embryo rescue in the cross *C. arietinum* × *C. pinnatifidum*. a. Pod formation after interspecific pollinations. b. Hybrid pod with dessicated ovule. c. Hybrid pod with a bigger but aborting ovule. d. Aborting ovule cultured to save the hybrid embryo. Note the emergence of the embryo from the ovule. e. Hybrid embryo isolated from the ovule and cultured.

Table 1. Interspecific hybridization between *Cicer arietinum* and wild species *C. pinnatifidum*

Genotypes	*C. pinnatifidum* ICCW 37					*C. pinnatifidum* ICCW 38				
	1	2	3	4	5	1	2	3	4	5
Kabuli										
92318	101	58	44	13	8	111	56	6	2	0
95311	134	35	17	0	0	108	50	4	2	0
ICCV 2	106	69	5	1	0	108	75	6	5	0
Desi										
ICCV-6	79	18	8	0	0	60	20	8	3	0
ICCV-10	218	113	29	12	9	122	21	15	1	0
ICCC-37	173	41	25	4	0	135	13	7	4	0
GL-769	47	22	9	1	0	NA	NA	NA	NA	NA
	858	356	137	31	17	644	235	46	17	0

1. No. of pollinations; 2. No. of pods set; 3. No. of ovules cultures; 4. No. of ovules with seedling emergence; 5. No. of plants obtained; NA – not available.

Three kabuli and four desi genotypes of chickpea (Table 1) were used as the female parent to cross with two accessions of *C. pinnatifidum*, a wild species from the tertiary gene pool of chickpea (Muehlbauer, 1993). Hybrid pods from the interspecific cross *C. arietinum* × *C. pinnatifidum* were obtained only after the application of the mixture of growth regulators (GA 75 mg l^{-1} + NAA 10 mg l^{-1} + KN 10 mg l^{-1}) to the base of the pollinated pistils to prevent premature pod abscission. Self pods on the plant was removed. Pods from cross pollinations were harvested between 18–25 days after pollination when the pod wall began to turn yellow. Ovules were cultured on ML-6 medium with 3% sucrose and with ZN at 1.0 mg l^{-1} and IAA at 0.25 mg l^{-1}.

After 30 days of ovule culture, growing embryos from selfed ovules as well as from the cross *C. arietinum* × *C. pinnatifidum* were transferred to fresh culture medium which consisted of ML-6 basal medium with IAA at 0.25 mg l^{-1} and ZN at 1.0 mg l^{-1}. Well developed plants with good root and shoot system were transferred to soil.

To check the hybrid plants for pollen fertility, anthers were squashed in 2% Acetocarmine, and observed under light microscope.

Polyacrylamide gel electrophoresis (PAGE) was carried out on 10% gel to isolate esterase isozymes. The gels were stained according to the method of Scandalios (1969).

Results

Ovules (immature seed) at 6 days after pollination were 2.0 mm in size and had embryos in the size range of 0.1–0.2 mm. Ovules at 8 days after pollination were 3.0–3.5 mm in size had an 0.3–0.4 mm early cotyledonary embryo. Ovules at 10 days after pollination were 4.5–5.0 mm and had embryos with well developed cotyledons and were 0.5 mm–0.7 mm in size.

Ovules in which embryo growth was later observed, were swollen and emergence of the embryo was observed at 35 days of culture from the ovules at 8, 10 and 12 DAP. None of the ovules from 6 DAP showed any of the above responses. Compared to 8 DAP, 10 DAP had more number of responding ovules with respect to the emergence of embryo. Of the 36 combinations of ZN and IAA used in the present study, ovules responded on those media that had IAA at 0.1–2.0 mg l^{-1} and ZN at 0.5–2.0 mg l^{-1} (Figure 1a, b and c). Absence of ZN or IAA in the culture medium did not promote the growth of the embryos in the ovules (Figure 1c). Maximum number of embryos emerged from the ovules when cultured on the medium with IAA at 0.25 mg l^{-1} and ZN at 1.0 mg l^{-1} (Figure 1b). These embryos had a well developed shoot and root system. Ovules did not respond when Zeatin in the culture medium was replaced by Kinetin.

Pod set in cross pollinations whenever obtained was only after the application of growth regulators. Not all the cross pollinations resulted in the formation of pods with fertilized seeds (Table 1). Many of the pods were morphologically normal (Figure 2a) but had aborted ovules within (Figure 2b). Such ovules which were dark brown and dried and which were less than 1.0 mm in size, were not cultured. Only immature seeds more than 1.5 mm in size and which were at different stages of abortion were cultured. The size of immature seeds ranged from 1.5 mm–7.0 mm (Figure 2b and c). The size of the embryo ranged from 0.1 mm to 4.0 mm. Fifty seven percent of the pollinations formed pods when kabuli type chickpea ICCV 92318 was crossed with *C. pinnatifidum* ICCW 37. Similarly 52% of the pollinations formed pods when desi type ICCV 10 was crossed with *C. pinnatifidum* ICCW 37. Of the 58 pods obtained in the cross ICCV 92318 × ICCW 37, only 44 immature seeds were obtained (Table 1). These immature seeds were cultured. Not all the immature seeds had the growth of the embryos within. Only embryos from 8 ovules germinated (Figure 2d and e) and gave rise to 4 pale-green and 4 albino plants. These were transferred to fresh medium to fecilitate their further growth (Figure 3a and c). After 75 days of culture 2 of the 4 albino seedlings turned pale-green and the 4 pale-green seedlings turned into normal green plants. Six green hybrid plants established well in soil (Figure 3d). They were acclamatized under controlled conditions of high humidity (78%) and at 26 °C for 16 hours of light and 8 hours of dark regime. In the cross ICCV 10 × ICCW 37, although 9 embryos with good root and shoot system were obtained, of which only one seedling was green. The eight seedlings did not survive the transfer to soil. Hence only one plant survived the transfer to soil.

Four of the 6 hybrids from the cross ICCV 92318 × ICCW 37 reached the flowering stage. The morphology of the hybrid leaf resembled the male parent (Figure 3b and d). The growth habit although intermediate had the tendency to spread, as seen in *C. pinnatifidum* ICCW 37, the male parent. The colour

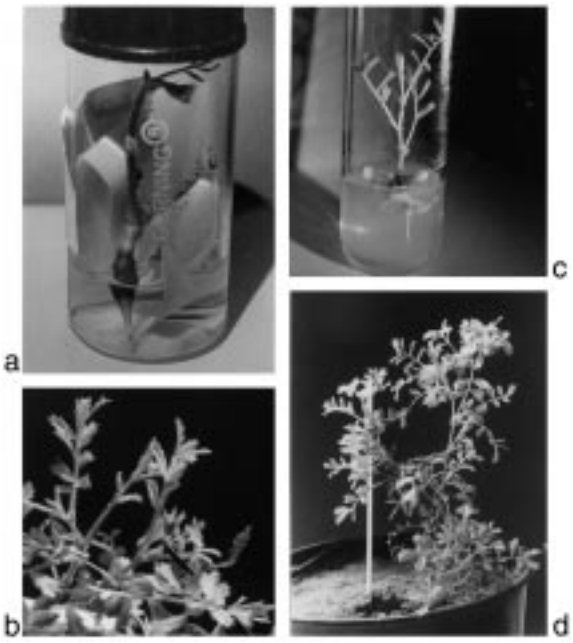


Figure 3. Embryo rescue in the cross *C. arietinum* × *C. pinnatifidum*, continued. a. Hybrid seedling growing in the liquid medium. b. Hybrid plant transferred to semi-solid medium. c. Hybrid plant transferred to soil. d. Close up of the hybrid plant. The arrow points at the fragile hybrid flower.

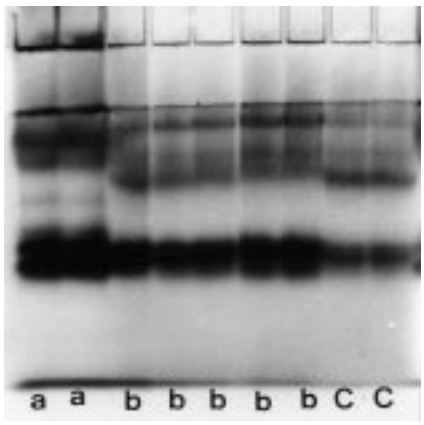


Figure 4. Esterase isozyme pattern of the parents and the hybrids. a. *C. pinnatifidum*. b. Hybrid plant. c. *Cicer arietinum*.

of the flower was pale violet, resembling the violet colour of the male parent and the flower colour of the female parent was white. The flowers were fragile and few in number (Figure 3b). The flowers had a prominent calyx with fragile petals. The petals did not open completely. All the hybrids were 100% pollen sterile. Esterase isozyme analysis of the parents and the hybrid plants showed two bands of the hybrid common to *C. pinnatifidum* and two bands of the hybrid common to CV. ICCV 92318.

Discussion

Until now only two wild species were crossable with cultivated chickpea (Muehlbauer, 1993). *Cicer reticulatum* which belongs to the primary gene pool together with cultivated *cicer* crosses freely with it to form mature seeds. The hybrid plants show least meiotic disturbance with high pollen fertility of more than 90% (Ladizinsky & Alder, 1976). *Cicer echinospermum* has been placed in the secondary gene pool, it crosses with cultivated chickpea to form mature seeds. Although some meiotic abnormalities are observed in the meiocites of the hybrid, pollen fertility ranges between 25–28% (Pundir & Mengesha, 1995). *C. pinnatifidum* is rightly placed in the tertiary gene pool of chickpea, because the cross *C. pinnatifidum* × *C. arietinum* is incompatible and the seeds abort before reaching maturity. Pollen of the hybrid plants are 100% sterile. These results show that the genomes of the two are not only distantly related may also be different.

Application of growth regulators to pollinated pistils is mandatory to obtain hybrid pods. Similarly in the experiments involving incompatible wild species in groundnut wide-crosses (Nalini & Sastri, 1985) without the application of gibberellic acid, pods were not obtained from cross pollinations.

One of the reasons for the formation of large number of pods as a result of pollination may be due to the stimulus provided by the process of pollination itself. This could be the reason for obtaining large number of pods with desiccated ovules. In many of the pods the embryo formed as a result of fertilization but aborted during the first week of pod formation. Hence many of the pods had ovules which were not only small (1–2 mm) but were also dry.

One of the deciding factors for obtaining a hybrid plant was a good root system depended upon the genotype of the female parent used in the crossing program. Hybrid plants with robust root system withstood the transfer to soil, and grew into a normal healthy plant. In the study by Badami et al. (1997) the female parent used in the crossing program was chickpea cultivar GL-769 which was crossed with two accessions of *C. pinnatifidum*, which were also used in the present study. Although hybrid shoots were obtained, Badami et al. (1997) were not able to root them *in vitro* and

transfer them to soil. In the present study, use of large number of accessions of cultivated chickpea as the female parent clearly showed the superiority of some genotypes to produce hybrid plants with robust root system.

The conversion of albino hybrid plants into green plants is due to the conversion of etioplasts into green chloroplasts. Badami et al. (1997) made TEM observations of the chloroplasts from hybrid albino shoots and hybrid green shoots of chickpea and showed that albino shoots had abnormal chloroplast structure with poorly developed thylakoids containing few and disorganised grana. They further showed that there was improvement in chloroplast structure in hybrid shoots which turned green in culture. According to Chory et al. (1991) light incubation conditions coupled with the cytokinin in the culture medium has a very important role to play in the conversion of etioplasts to chloroplasts in the mutants of *Arabidopsis thaliana*.

The above-mentioned embryo rescue technique can be used to save aborting embryos from cross between chickpea and the other wild species which were until now not crossable. In *Vicia* and *Trifolium* wide crosses in-ovulo embryo rescue techniques have yielded hybrids which were not possible before (Lazaridou et al., 1993; Przywara et al., 1989).

The present investigation has opened up avenues to introgress genes from incompatible wild annual as well as perennial *Cicer species.*

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