View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by ICRISAT Open Access Repository

Plant Cell, Tissue and Organ Culture (2006) 85: 197–204
DOI 10.1007/s11240-005-9071-1
© Springer 2006

Embryo rescue and plant regeneration *in vitro* of selfed chickpea (*Cicer arietinum* L.) and its wild annual relatives

H.J. Clarke[1,*], J.G. Wilson[1], I. Kuo[1], M.M. Lülsdorf [2], N. Mallikarjuna[3], J. Kuo[4] & K.H.M. Siddique[1]

[1]*Centre for Legumes in Mediterranean Agriculture (CLIMA), The University of Western Australia, 35 Stirling Hwy, Crawley, WA 6009, Australia;* [2]*Crop Development Centre, University of Saskatchewan, Saskatoon, SK S7N 5A8, Canada;* [3]*International Crops Research Institute for the Semi Arid Tropics (ICRISAT), Patancheru, AP 502 324, India;* [4]*Centre for Microscopy and Microanalysis, The University of Western Australia, 35 Stirling Hwy, Crawley, WA 6009, Australia (*requests for offprints: Fax: +61-8-64881140; E-mail: hclarke@cyllene.uwa.edu.au)*

Received 11 November 2005; accepted in revised form 13 December 2005

Key words: grain legume improvement, interspecific hybridisation, tissue culture, wild relatives

Abstract

The main constraint to the transfer of desired traits into cultivated chickpea from wild *Cicer* relatives is the presence of post-zygotic barriers which result in abortion of the immature embryo following interspecific hybridisation. Rescue of hybrid embryos *in vitro* and regeneration of hybrid plantlets could allow chickpea breeders to transfer desirable traits from wild relatives of chickpea. The development of embryo rescue techniques using selfed chickpea and selfed wild relatives is being used as a first step to protocols for wide hybrids. Optical microscopy studies of embryogenesis, in both selfs and hybrids, identified deleterious changes in the fertilised hybrid seed as early as 2–4 days after pollination in some crosses. These observations suggest that the appropriate time to rescue chickpea × *C. bijugum* hybrids is at the early globular stage of embryogenesis (2–7 days old), which requires the development of a complex tissue culture medium. In contrast hybrids between chickpea × *C. pinnatifidum* abort later (up to 15–20 days old) at the heart-shaped or torpedo stages, and are easier to rescue *in vitro*. Genotype also plays a significant role in the ability of immature selfed ovules to germinate *in vitro*. In this paper we report on the optimisation of protocols for rescueing immature embryos using selfed chickpea and its wild relatives *in ovule*, and subsequently to regenerate plantlets.

Introduction

Wild relatives of chickpea within the genus *Cicer* can offer a broad range of genes for resistance to pest and disease and tolerance to abiotic stresses to the domesticated crop (reviewed by Croser et al., 2003). Although the wild *Cicer* species are recognised as a valuable genetic resource, they have not been widely exploited in chickpea breeding. Introgression of genes has been limited to two annual species most closely related to chickpea and falling within the primary gene pool. Cyst nematode resistance was successfully introgressed from *C. reticulatum* (Singh et al., 1996; Malhotra et al., 2002) and root lesion nematode resistance and phytophthora resistance from *C. echinospermum* (Knights et al., 2002). More recently, a range of genes for disease resistance and superior productivity was introgressed into chickpea from *C. reticulatum* (Singh et al., 2005). The major

constraint to full utilisation of the other wild relatives is the incompatability between chickpea and the remaining 40 known species of *Cicer* which fall into the secondary and tertiary genepools.

Chickpea and its wild annual relatives are diploid ($2n = 2x = 16$) self-pollinated species. Both pre-zygotic and post-zygotic barriers have been identified in interspecific hybridisations among *Cicer* species. Incompatability before fertilisation was reported in several crosses due to failure of pollen function at different levels from pollen germination, bursting or twisting of pollen tubes, to inhibition of pollen tube entry into the ovule (Swamy and Khanna, 1991; Singh et al., 1999). Incompatability following fertilisation is even more widespread among the annual species, with barriers to hybridisation with chickpea reported due to both embryo and endosperm breakdown (Bassiri et al., 1987; Ahmad et al., 1988; Swamy and Khanna, 1991; Ahmad and Slinkard, 2003; Ahmad and Slinkard, 2004). When a pod forms it becomes yellow during early stages of development and is abscised from the mother plant.

In this situation, embryo rescue *in vitro* may provide a means to overcome the abortion of the hybrid embryo *in situ*. Promising results have already been achieved in the rescue of hybrid ovules between chickpea and *C. pinnatifidum* and regeneration of plantlets (Mallikarjuna, 1999). An international collaborative effort is now being made to further develop robust *in vitro* culture procedures to overcome incompatability in a wider range of *Cicer* species and genotypes.

Hence, the aims of this study were to examine embryogenesis in selfed and hybridised ovules; to develop robust techniques to rescue immature embryos *in vitro* using selfed chickpea ovules; to regenerate plantlets from rescued chickpea ovules; and to apply the best techniques to rescue interspecific hybrids.

Our hypothesis is that optimisation of genotype, timing for rescue of the ovule and culture medium will enhance survival of the embryo and regeneration of healthy plantlets.

Materials and methods

Germplasm, plant growth conditions and hybridisation

Plants were grown in a controlled temperature glasshouse (20 °C day/15 °C night) located on the Crawley campus of the University of Western Australia (31° 59′ S; 115° 49′ E). Detailed methods for pot studies are described in Clarke and Siddique (2004). A range of chickpea cultivars and wild *Cicer* accessions (Table 1) were sown at two weekly intervals to ensure a continual source of flowers for hybridisations and selfed ovules for tissue culture experiments. For hybridisation, buds of chickpea were emasculated before anthesis, and the stigma was hand pollinated with fresh pollen from *C. bijugum* or *C. pinnatifidum* to create the interspecific hybrids. Plant growth regulators were applied to the petiole of the crossed flower to delay abscission (Mallikarjuna, 1999). Older selfed pods were removed to divert photo-assimilates to the crossed flowers.

Microscopy

Flowers and pods were harvested from mother plants at 0–16 days after pollination. To examine pre-zygotic barriers to hybridisation, style samples were stained with 0.1% aniline blue in deionised water and pollen tubes were observed using UV fluorescence. To examine embryogenesis and post-zygotic barriers to hybridisation, ovules were dissected from immature pods aged from 2 to

Table 1. Chickpea genotypes and annual wild *Cicer* species used in the study

Species	Genotype	Description
C. arietinum	Sonali	Desi type chickpea cultivar
	Rupali	Desi type chickpea cultivar
	Ted57Q	Desi type chickpea advanced breeding line (derivative of chickpea × *C. echinospermum*)
C. bijugum	ILWC7	Wild annual, secondary gene pool
	ILWC70	Wild annual, secondary gene pool
	ILWC84	Wild annual, secondary gene pool
C. pinnatifidum	ILWC88	Wild annual, secondary gene pool

10 days after pollination (both self- and cross-pollinated), fixed, dehydrated in an ethanol series and embedded in Spurr's resin. Longitudinal serial sections (0.5 μm) of the embryo and surrounding tissue of the ovule were cut, stained with 0.5% toluidine blue pH 9.0, and examined under bright field microscopy on a Zeiss Axioshot.

Embryo rescue in ovule

Pods were removed from mother plants and surface sterilised in 1% bleach for 15 mins, rinsed thoroughly with sterile deionised water and transferred to sterile petri dishes, under aseptic conditions. Ovules were dissected from the pods and transferred to filter paper bridges in 30 ml polycarbonate tubes containing 5 ml of liquid culture medium. Care was taken to ensure that the cut surface of the funiculus was in contact with the filter paper and the culture medium. Selfed chickpea ovules were used to investigate the role of (i) genotype, (ii) age of the ovule, (iii) size of the ovule and (iv) culture medium. The control medium used in the experiments was ML6 medium with 90 g l^{-1} sucrose (Kumar et al., 1988), 1 mg l^{-1} zeatin and 0.25 mg l^{-1} indole acetic acid (Mallikarjuna, 1999). Ovules were cultured in the light with a 16:8 h photoperiod at 25 °C. Ovule size was measured longitudinally. Ovule colour, number of embryos and a description of the embryo was recorded after 2, 4 and 8 weeks.

Pods from the interspecific crosses were maintained on the mother plants as long as possible, but were rescued when they showed any signs of yellowing and iminent abortion. Hybrid ovules were dissected from sterilised pods and cultured in ML6 medium with 1 mg l^{-1} zeatin and 0.25 mg l^{-1} indole acetic acid.

'Germination' of selfed embryos and regeneration of plantlets

Embryos were dissected from the ovule when they broke through the seed coat, and were transferred to a fresh tube, with a filter paper bridge, containing 5 ml of ML6 without plant growth regulators. Once germinated, plantlets were micropropagated in 250 ml polycarbonate containers. The multiplication medium was MS (Murashige and Skoog, 1962), supplemented with B5 vitamins (Gamborg et al., 1968), 30 g l^{-1} sucrose, 0.1 mg l^{-1} benzyl amino purine and 0.01 mg l^{-1} naphthalene acetic acid and 9 g l^{-1} agar (modified from Pigeaire et al., 1997) for 28 days. Shoots were subcultured to a similar medium supplemented with 3 mg l^{-1} indole butyric acid for root induction for 14 days. Once roots formed, the plantlets were cultured on the same medium without plant growth regulators and 4.5 g l^{-1} agar for 7 days, to encourage root growth prior to transfer to the glasshouse (Chapple, unpublished data).

Results

Barriers to hybridisation

Pollen and styles were compatible in all of the hybridisations between the cultivars and the accessions of *C. bijugum* and *C. pinnatifidum* used in this study. Pollen from the wild species germinated successfully and pollen tubes were visible in the ovary within 24 h of hand pollination (Figure 1a).

Sections through immature cross-fertilised ovules show the following features. Hybrid ovules with heart-shaped or later embryos were rare. Those sectioned with embryos had a small embryo sac, a very immature globular embryo and elongated suspensor cells (Figure 1b). Most hybrid ovules showed no swelling of the embryo sac, and no embryo was present. Cell death was seen in the integuments of some aborted hybrid ovules. Endosperm was clearly visible in ovules with an embryo, and in some ovules without an embryo. Selfed ovules had an embryo sac containing endosperm, spherical suspensor cells, globular (Figure 1c) and heart-shaped (Figure 1d) embryos.

Timing for rescue of embryos

No single stage of development in cross-pollinated samples was identified as most susceptible to abortion. Examination of sections through cross fertilised ovules of different ages showed that most *C. bijugum* hybrids aborted at very early stages of embryo development, 4–8 days after pollination, compared to *C. pinnatifidum* hybrids, which reached the heart-shaped stage from 10 to 12 days after pollination and onwards. Some *C. pinnatifidum* hybrids developed *in situ* to the torpedo stage, and could be left on the mother plant for up to 22 days.

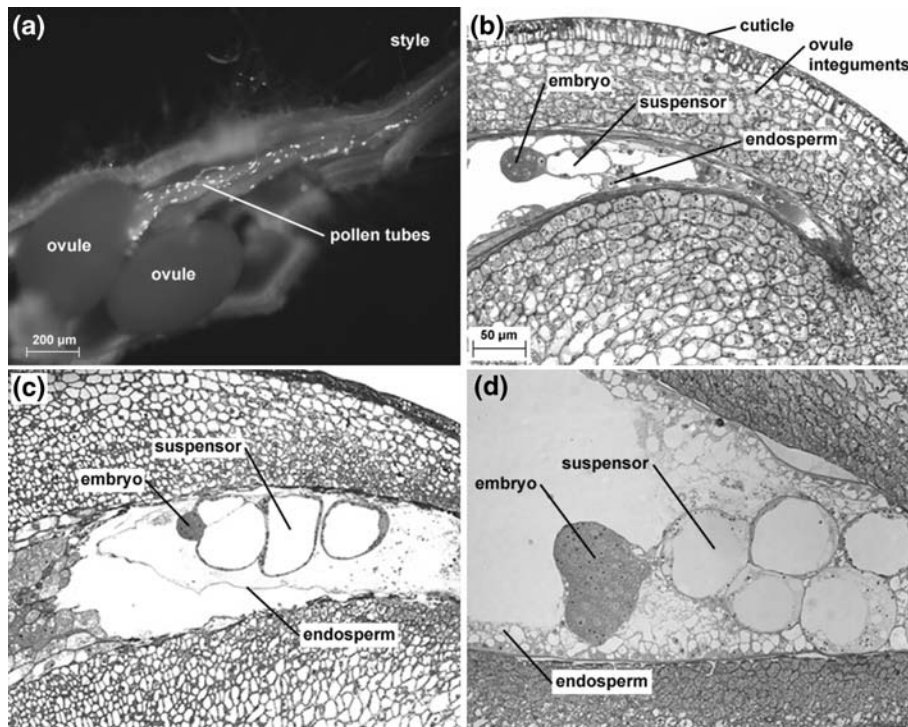

Figure 1. Barriers to interspecific hybridisation in *Cicer in situ*. Pre-zygotic: (a) pollen tubes of *C. bijugum* (wild) grow down the style to fertilise ovules of *C. arietinum* (chickpea) within 24 h. Post-zygotic: (b) 7 day old hybrid globular embryo; hybrid embryos rarely develop beyond 8 days old and none reach maturity if left *in situ*; (c) 6 day old selfed globular embryo; (d) 9 day old selfed heart-shaped embryo. Median longitudinal section of developing ovules (b–d).

A study of the response of immature selfed ovules following rescue *in vitro* (Figure 2) demonstrated a positive correlation ($p < 0.001$) between germination of an embryo and the age of the rescued ovule. Germination ranged from 0 in 5-day old ovules to 43% in 12-day old ovules. There was also a positive correlation ($p < 0.001$) between germination and the actual size of the rescued selfed ovule. Germination was only 10% in selfed ovules which measured 3 mm at the time of rescue, compared to 40% germination in 8 mm ovules.

In selfed chickpea there is a direct relationship between the size of an ovule and the number of days after pollination ($p < 0.001$), but our preliminary observations suggest that this correlation is less reliable in cross-pollinated ovules.

Variation between genotypes

There was wide variation between the genotypes examined in the ability of their immature selfed ovules to germinate after rescue *in vitro* (Figure 2). There was a significant difference between cultivated chickpea and its wild relative *C. bijugum*. Among the cultivated chickpea germplasm, there was no significant difference between the genotypes examined, which included an advanced breeding line (Ted57Q) derived from a chickpea × *C. echinospermum* interspecific cross. Thirty eight percent of Sonali, 31% Rupali and 30% Ted57Q germinated within 4 weeks. There was, however, a significant difference between the two accessions of *C. bijugum*: 21% of ovules germinated in ILWC7 compared to only 8% in ILWC84. None of the cross-pollinated ovules gave rise to germinated hybrid embryos.

Regeneration of plantlets and transfer to the glasshouse

A good success was achieved in the regeneration of plantlets from rescued selfed chickpea ovules (Figure 3). Regeneration was improved by excising the emerging embryo from the ovule coat and placing it in direct contact with the filter paper

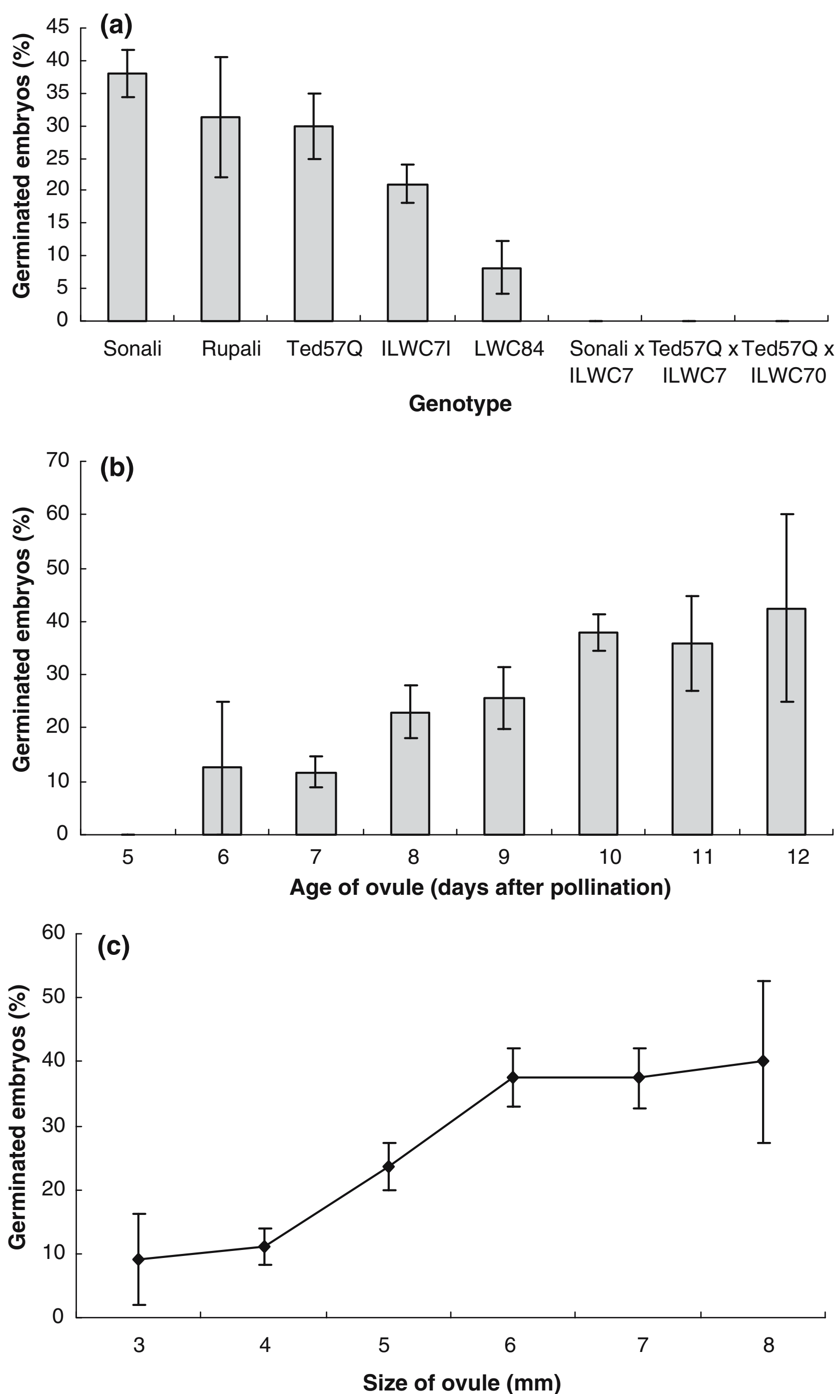

Figure 2. Response of immature ovules following rescue and culture on ML6 medium containing 90 g l^{-1} sucrose, 1 mg l^{-1} zeatin and 0.25 mg l^{-1} indole acetic acid for 4 weeks. (a) Genotypic variation among chickpea cultivars, wild accessions and hybrids (10 day old); (b) ovules at different ages rescued from the mother plant; and (c) ovules of different sizes rescued from the mother plant. An ovule was scored as germinated when the embryo erupted through the integuments of the coat (mean ± SE).

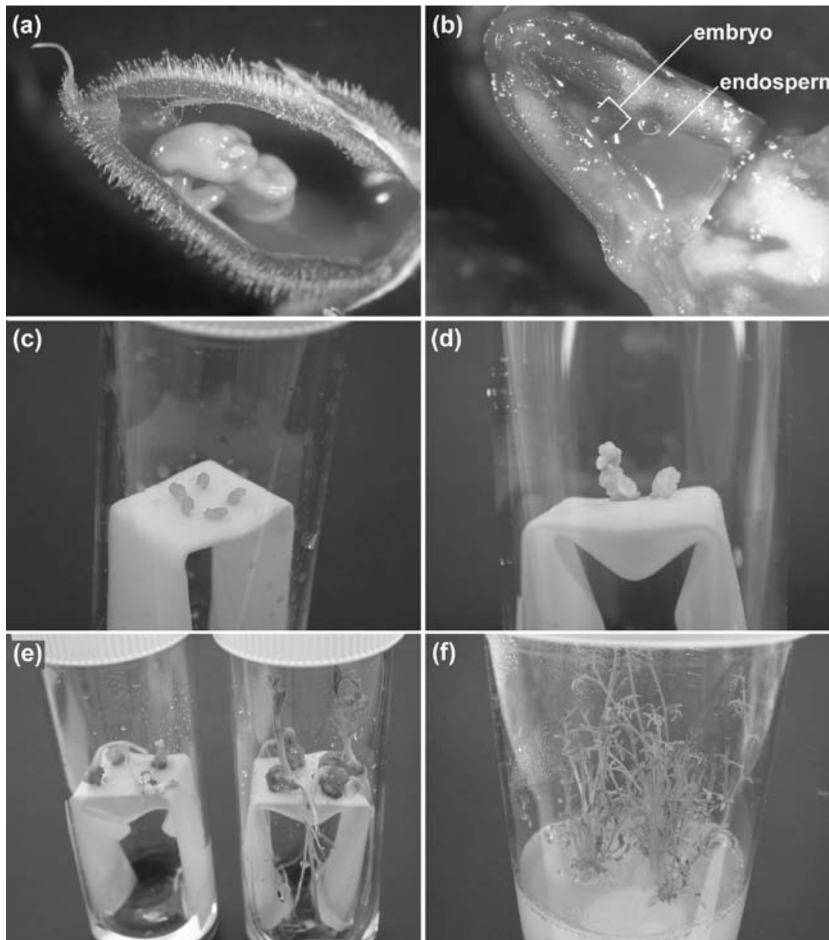

Figure 3. Rescue *in ovule* and culture of selfed chickpea embryos 8–10 days after pollination. (a) Dissected pod showing ovules *in situ*; (b) dissected ovule showing stage of development of an 8 day old selfed embryo and endosperm; (c) ovule rescue *in vitro* on filter paper bridges in liquid ML6 culture medium; (d) growing embryo erupts through the wall of the ovule at 3 weeks; (e) hypocotyl and/or roots develop at 6–12 weeks; (f) shoots are micropropagated on solid MS medium, supplemented with B5 vitamins and plant growth regulators for a further 4 weeks.

bridge and medium in a fresh culture tube. Callus formation was reduced by omitting plant growth regulators. Multiplication of shoots, root induction and transfer to soil was efficient and reproducible for selfed chickpea and selfed *C. pinnatifidum*. On the other hand, shoots of *C. bijugum in vitro*, derived from rescued immature selfed ovules, were less amenable to rooting and had a lower survival rate in the glasshouse.

Discussion

A significant outcome of this research is our observations that viable embryos develop following interspecific hybridisation between cultivated chickpea and two distantly related species of its secondary genepool. Sections were obtained through such hybrids in 7-day old cross-pollinated ovules. Secondly, both genotype and the age of the developing ovule were found to be important factors in successful embryo rescue *in vitro*. The development of embryo rescue techniques using selfed chickpea and selfed wild relatives was used as a first step to developing robust protocols for wide hybrids. This approach allows us to use larger uniform samples and perform many experiments without the need for crosses. Results described in this paper support our hypothesis that optimisation of genotype, timing for rescue of the ovule and the medium for culture *in vitro* will improve survival of the embryo and regeneration of healthy plantlets.

In this study no pre-zygotic barriers to pollen germination or tube growth were found in the wide crosses between chickpea and its wild relatives *C. pinnatifidum* and *C. bijugum*. Unfortunately, incompatibility occurred during early embryo development *in situ*. No particular stage of embryo development was identified as most sensitive to abortion, but examination of sections through immature ovules demonstrated that *C. bijugum* hybrids aborted early after pollination *in situ*; and it was rare to find any heart-shaped embryos (e.g. at 10 days). In comparison, *C. pinnatifidum* hybrids remained viable on the mother plant for longer (15–22 days). These findings support the strategy to culture cross-pollinated ovules *in vitro* as a means to rescue the hybrid embryos before they abort.

The absence of pre-zygotic barriers in our wide *Cicer* crosses is supported by previous observations of Bassiri et al. (1987) and Ahmad et al. (1988) who report pollen of these wild species germinating in styles of chickpea, as well as many reports of pod set at low frequency followed by abortions (reviewed by Croser et al., 2003).

Our microscopy studies reveal some abnormalities within the cross-pollinated ovule. We observed some differences in the shape of embryo suspensor cells and the integuments of the ovule wall which may represent post-zygotic barriers in chickpea × *C. bijugum* crosses. In addition, the presence of healthy endosperm in the aborting hybrids indicates that double fertilisation has taken place. Previous reports suggest that deficient endosperm (Geerts et al., 2002; Ahmad and Slinkard, 2004) or degeneration of endosperm (Stamigna et al., 2000) is the cause of abortion in some legumes. In hybrid ovules of chickpea × *C. bijugum*, endosperm breakdown has been observed (Singh et al., 1999); this was not the case in our study. When a hybrid embryo was found, the cells of the embryo, as well as the endosperm, were viable and actively dividing. The results of these studies confirm that embryo rescue *in vitro* will facilitate wide crosses between chickpea and its wild relatives.

The variable response of ovules following culture, depending on the age and size of the ovules, is an important issue to consider for embryo rescue. Our experiment clearly showed that selfed ovules which remain on the mother plant for 12 days, in which the embryo has reached the heart-shaped stage of development before rescue *in vitro*, have a 40% chance of germination and giving rise to a plantlet. In contrast, a very young ovule (e.g. 5 days) with a very immature proembryo or globular embryo will not survive *in vitro* on our best current culture medium. The direct positive correlation between stage of embryo development and success of ovule rescue *in vitro* has also been reported in green beans (Geerts et al., 1999). This issue is a major limiting factor for successful embryo rescue of interspecific hybrids, particularily between chickpea and *C. bijugum* which rarely develops to heart-shaped stage on the mother plant.

Significant genotypic variation in the germination response of immature ovules *in vitro* is an important finding of this study. The identification of responsive genotypes, in particular chickpea cultivars useful as the mother plant in an interspecific cross, is a critical step in method development. Such cultivars are now being used to further optimise the culture medium needed to support growth of very immature ovules. Genotypic variation in the response of rescued ovules and embryos to *in vitro* culture is common in most species, including, for example, cereals (Baum et al., 1992) and legumes (Mallikarjuna, 2003). Genotype of the pollen donor, as well as the mother plant, also plays a crucial role in the success of hybridisation, and is the subject of current experiments.

Rescue of selfed ovules also allowed us to test media for multiplication of shoots and induction of roots, and to develop techniques for transfer of plantlets from tissue culture in controlled conditions to soil in the glasshouse. The methods described here were successful for the genotypes that we examined in Australia. Grafting of tissue-cultured plantlets is also being examined as an alternative for some genotypes which are more difficult to root.

We conclude that embryo rescue is a valuable tool for researchers and breeders to create interspecific hybrids between chickpea and distant wild relatives, crosses which were considered impossible until recently. A robust protocol for the rescue of any *Cicer* germplasm, which the breeder might select for crosses, would be ideal. But at the present early phase of method development, genotypic variation in germination response is an important factor to consider. A suitable culture

medium was previously developed for the rescue of chickpea × *C. pinnatifidum* hybrids (Mallikarjuna, 1999), which can survive on the mother plant for longer than 12 days, by which time the embryo is already well developed to the heart-shaped stage. The next step in our research is to optimise the culture medium to support growth and development of very immature ovules to enable the rescue of chickpea × *C. bijugum* hybrids which abort on the mother plants within a few days of fertilisation. For routine rescue of these hybrids, we anticipate that a complex culture medium will be required to support embryo growth from such very early stages of development. Further histological studies will also examine the role of mother tissue in the ovule in the abortion of hybrid embryos. We are also investigating ways to maintain the hybrids on the mother plant as long as possible before rescue.

Acknowledgements

This work is supported by the Grains Research and Development Corporation of Australia and CLIMA. We are grateful to the curators of genebanks, at the International Center for Agricultural Research in Dry Areas in Syria, the International Crops Research Institute for the Semi Arid Tropics in India and the Australian Temperate Field Crops Collection in Australia, for providing germplasm used in the study.

References

Ahmad F & Slinkard AE (2003) Limitations to bridge-species facilitated alien gene transfers in chickpea: pre-fertilization events. J. Genet. Breed. 57: 69–74

Ahmad F & Slinkard AE (2004) The extent of embryo and endosperm growth following interspecific hybridization between *Cicer arietinum* L. and related annual wild species. Genet. Resources Crop Evol. 51: 765–772

Ahmad F, Slinkard AE & Scoles GJ (1988) Investigations into the barrier(s) to interspecific hybridization between *Cicer arietinum* L. and eight other annual *Cicer* species. Plant Breed. 100: 193–198

Bassiri A, Ahmad F & Slinkard AE (1987) Pollen grain germination and pollen tube growth following *in vivo* and *in vitro* self and interspecific polinations in annual *Cicer* species. Euphytica 36: 667–675

Baum M, Lagudah ES & Appels R (1992) Wide crosses in cereals. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43: 117–143

Clarke H & Siddique KHM (2004) Response of chickpea genotypes to low temperature stress during reproductive development. Field Crops Res. 90: 323–334

Croser JS, Ahmad F, Clarke HJ & Siddique KHM (2003) Utilization of wild *Cicer* in chickpea improvement – progress, constraints and prospects. Aust. J. Agric. Res. 54: 429–444

Gamborg OL, Miller RA & Ojima K (1968) Nutrient requirement of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151–158

Geerts P, Mergeai G & Baudoin JP (1999) Rescue of heart-shaped embryos and plant regeneration of *Phaseolus polyanthus* Greenm. and *Phaseolus vulgaris* L. Biotechnol. Agron. Soc. Environ. 3: 141–148

Geerts P, Toussaint A, Mergeai G & Baudoin JP (2002) Study of early abortion in reciprocal crosses between *Phaseolus vulgaris* L. and *Phaseolus polyanthus* Greenm. Biotechnol. Agron. Soc. Environ. 6: 109–119

Knights EJ, Brinsmead RB, Fordyce M, Wood J, Kelly AG & Harden S (2002) Use of wild relative *Cicer echinospermum* in chickpea improvement. In: Mc Comb JA (ed) 'Plant Breeding for the 3rd Millenium', 12th Australasian Plant Breeding Conference. Australasian Plant Breeding Association Inc, Perth Western Australia (pp. 150–154)

Kumar AS, Gamborg OL & Nabors MW (1988) Plant regeneration from cell suspension cultures of *Vigna aconitifolia*. Plant Cell Rep. 7: 138–141

Malhotra RS, Singh KB, Di Vito M, Greco N & Saxena MC (2002) Registration of ILC 10765 and ILC 10766 chickpea germplasm lines resistant to cyst nematode. Crop Sci. 42: 1756

Mallikarjuna N (1999) Ovule and embryo culture to obtain hybrids from interspecific incompatible pollinations in chickpea. Euphytica 110: 1–6

Mallikarjuna N (2003) Wide hybridisation in important food legumes. In: Jaiwal PK & Singh RP (eds) Improvement strategies of leguminosae biotechnology. Kluwer, Dordrecht (pp. 155–170)

Murashige T & Skoog FG (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Pigeaire A, Abernethy D, Smith PM, Simpson K, Fletcher N, Lu C-Y, Atkins CA & Cornish E (1997) Transformation of a grain legume (*Lupinus angustifolius* L.) via *Agrobacterium tumefaciens*-mediated transfer to shoot apices. Mol. Breeding 3: 341–349

Singh KB, Di Vito M, Greco N & Saxena MC (1996) Registration of ILWC 292, a chickpea cyst nematode-resistant germplasm of *Cicer reticulatum* Ladiz. Crop Sci. 36: 1421–1422

Singh NP, Singh A & Asthana AN (1999) Studies on interspecific crossability barriers in chickpea. Ind. J. Pulses Res. 12: 13–19

Singh S, Gumber RK, Joshi N & Singh K (2005) Introgression from wild *Cicer reticulatum* to cultivated chickpea for productivity and disease resistance. Plant Breed. 124: 477–480

Stamigna C, Crino P & Saccardo F (2000) Wild relatives of chickpea: multiple disease resistance and problems to introgression in the cultigen. J. Genet. Breed. 54: 213–219

Swamy AVSR & Khanna VK (1991) Pollen grain germination and pollen tube growth following interspecific pollinations in *Cicer*. Sabrao Journal 23: 137–145