Indian J. Genet., 67(4): 414-417 (2007)

Short Communication

In vitro regeneration of *Cicer arietinum* L. from callus cultures

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(Received: August 2006; Revised: October 2007; Accepted: October 2007)

Grain legumes are intractable-to-regenerate *in vitro* which is a major problem to be solved before transgenic methods can be used for the improvement of legumes. Direct regeneration or regenerant differentiation via callus cultures and subsequent regeneration of whole plants are essential for plant improvement through biotechnological interventions.

Cicer arietinum (chickpea) is an important pulse crop, grown mainly for its protein content. Micropropagation and production of somaclonal variants in them and their wild relatives could be very useful for effective improvement. In chickpea, caulogenesis, embryogenesis and also plantlet formation has been reported employing different explants [1-6], but reproducible high frequency differentiation of regenerants has been mostly sporadic inspite of repeated attempts. The present communication reports a system for achieving high-frequency shoot multiplication via indirect organogenesis in *Cicer arietinum*.

Seeds of *Cicer arietinum* L. were surface sterilized using 0.2% $HgCl_2$ solution for 30 min, thoroughly washed with sterile water and were incubated, one seed per 20 ml of modified semisolid White's medium [7] in complete dark for germination. Shoot apex (0.5 to 1 cm) explants were excised from a 10 day old aseptically grown seedlings and cultured on modified MS medium [8] supplemented with NAA (0.25 mg/l), BAP (0.5 mg/l) and AdS (10 mg/l). The cultures were established separately on media maintained at 25 ± 1°C with a photoperiod of 16/8h light-dark regimes (2500 lux fluoresent light). The cultures were transferred to the fresh medium at regular 4 week intervals after the initial culture period of 20 days. Five to ten replicates with one explant were incubated and each experiment was repeated thrice. Effect of different concentrations and combinations of BAP, NAA and other reduced nitrogen sources like L-glutamine, L-tyrosine and CH were seen on shoot bud differentiation. Later, 0.25 mg/l Zeatin and 2iP was substituted for BAP in a treatment with 0.1 mg/l NAA.

Portions of somatic callus showing initiation of shoot bud differentiation were excised and subcultured onto basal medium containing BAP and kinetin to stimulate shoot development and proliferation. Shoots measuring 3-4 cm in length were excised and cultured on medium lacking growth regulators or containing NAA at different concentrations. Healthy plantlets with 2 cm long roots were individually removed from the culture tubes and transplanted into paper cups containing sterilized potting mixtures (garden soil + compost; 3:1).

The responses of callus tissue obtained from shoot tip explants of chickpea to media containing various growth regulators and other supplements are summarized in Table 1. The three step culture system for successful regeneration of chickpea via callus was as follows:

1. Callus was initiated on the modified MS basal medium supplemented with BAP (0.05 mg/l), NAA (0.1 mg/l) plus AdS (10 mg/l) and L-glutamine (25 mg/l).
2. For shoot bud differentiation, callus was transferred to a medium having NAA (0.1 mg/l), BAP (0.5 mg/l) and L-tyrosine (30 mg/l).
3. Roots were induced from isolated shoots in a rooting medium containing 0.1 mg/ls NAA.

Swelling followed by initiation of callus took place after 2 weeks of incubation and it continued to increase

Table 1. The effect of various growth regulators and growth supplements on induction of shoot bud differentiation in somatic callus of chickpea (*Cicer arietinum* L.). Data recorded after 40 days of incubation in each treatment in a modified MS medium

Growth regulators (mg/l)	NAA = 0.1 BAP = 0.05 AdS=10	0.1 0.1	0.1 0.5	0.25 0.5	0.1 2iP = 0.25	0.1 Z = 0.25
Tyrosine						
(20)	+++dark green*			+++green*	++brown	++dirty brown
(30)	-	++green•	+++green*			-
Glutamine						
(25)	+++dark green*	-	-	++ green*	-	-
CH						
(200)	+++dark green*	-	-	+++green*	-	-
(300)	-	++green'	++brown	++brown	-	-
Liquid & filter paper bridge CH (300)	-	Callus turned blackish brown	-	-	-	-

•Fresh green friable callus. Initiation of green shoot bud like projections appeared during the first 4-6 weeks of subculturing.
*Profuse swelling of callus tissue with multiple shoot formation, followed by good shoot development.
The number of + sign denotes degree of callus growth.

in amount when sub-cultured on the same basal medium. Callus pieces were excised from the freshly induced callus and subcultured on the same medium for further proliferation (Fig. 1A).

Callus was greenish and friable, grew satisfactorily in the medium supplemented with BAP (0.1 mg/l), NAA (0.1 mg/l), AdS (10 mg/l) plus L-Tyrosine (30 mg/l). In the medium containing reduced concentration of NAA (0.1 mg/l), BAP (0.01 mg/l) and supplements of L-glutamine (25 mg/l) and L-tyrosine (20 mg/l) resulted in shoot bud differentiation in ca. 60% cultures (Fig. 1B) after 40 days of inoculation (Table 2).

The morphogenic portions of callus each containing 5-8 shoot buds when cultured in a treatment having 0.05 mg/l BAP, 0.1 mg/l NAA and 200 mg/l CH, resulted in growth of shoot buds and proliferation and development of shoots (Fig. 1C). Glandular epidermal trichomes normally present on chickpea leaves were a clearly visible indicator of shoot initiation. The shoots grew vigorously with well developed foliage. Vigorously growing shoots with a good number of developed leaves were isolated and rooted 90% in 0.1 mg/1 NAA without any intervening callus formation in 20 days (Fig. 1D). A total of ca. 400 callus regenerated plantlets were rooted and successfully established.

The use of cytokinin, BAP, has been widespread for the induction of organogenesis in legumes and in most of the studies, it has been exclusively used. A few studies suggest that BAP can be the more effective cytokinin than others but comprehensive studies comparing the effectiveness of BAP with that of the other cytokinins are lacking in legumes [9]. In the present study, BAP effectively induced organogenesis, while Z and 2iP did not. Similar observations have been reported in pea where both BAP and Kn induced regeneration although with differing effectiveness, while Z and 2iP have been ineffective [10] while in Indigo [11] both Kn and Z, were ineffective in inducing regeneration.

Multiple shoot bud induction was obtained in low levels of NAA (0.1 mg/l) in combination with higher as well as lower levels of BAP (0.01-0.5 mg/l) having additional supplements like L-glutamine (25 mg/l) and L-tyrosine (20 mg/l). This effective hormonal combination evoked more than 20 shoot buds from a single explant. In contrast to with earlier reports, the responses in the optimum treatment, (0.05 mg/l BAP, 0.1 mg/l NAA, 10 mg/l AdS and 200 mg/l CH) were reproducible to the tune expected in the biological systems.

The use of unconventional methods for broadening the genetic base in legumes has assumed considerable significance and hence, efforts continue to be made in

Table 2. Efficacy of MS medium fortified with growth regulators and different adjuvants on adventitious shoot regeneration from shoot tip derived callus

Growth Regulators	Adjuvants (mg/l)				% Response	No. of shoots/ culture (mean + SE)
BAP	NAA	Tyrosine	Glutamine	CH		
0.01	0.1	20	25		60	33.10 ± 0.79
0.1	0.1	30			45	5.80 ± 0.29
0.5	0.1	30			45	7.80 ± 0.39
0.5	0.25	20			40	5.30 ± 0.30
0.05	0.1	25			50	8.50 ± 0.34
0.5	0.25		25		48	10.50 ± 0.34
0.05	0.1			200	50	14.90 ± 0.31
0.1	0.1			300	40	6.00 ± 0.66
0.5	0.25			200	45	7.70 ± 0.30

The data taken after 40 days of incubation comprises of 10 replicates each.

search of methods to generate genetic variation without drastically altering the genetic back ground. It is concluded that the efficient multiple shoot formation, induced in the present investigation in *Cicer arietinum* will be useful in direct gene transfer studies using the method of particle bombardment and subsequent regeneration of transgenic plants.

A protocol for an efficient plant regeneration of *Cicer arietinum* L. via organogenesis from callus has been developed. Callus was initiated from shoot tip explants in a modified MS medium supplemented with a number of growth adjuvants. Shoot bud differentiation was observed in the treatment containing BAP (0.5 mg/l), NAA (0.25 mg/l), L-glutamine (25 mg/l), L-tyrosine (20 mg/l) and AdS (10 mg/l). Caulogenic portions of callus with shoot buds when cultured in the modified MS medium containing 0.1 mg/l BAP, 0.1 mg/l NAA and 30 mg/l L-tyrosine resulted into proliferation of shoots and their development. The protocol developed may be utilized for genetic transformation studies in this large seeded legume.

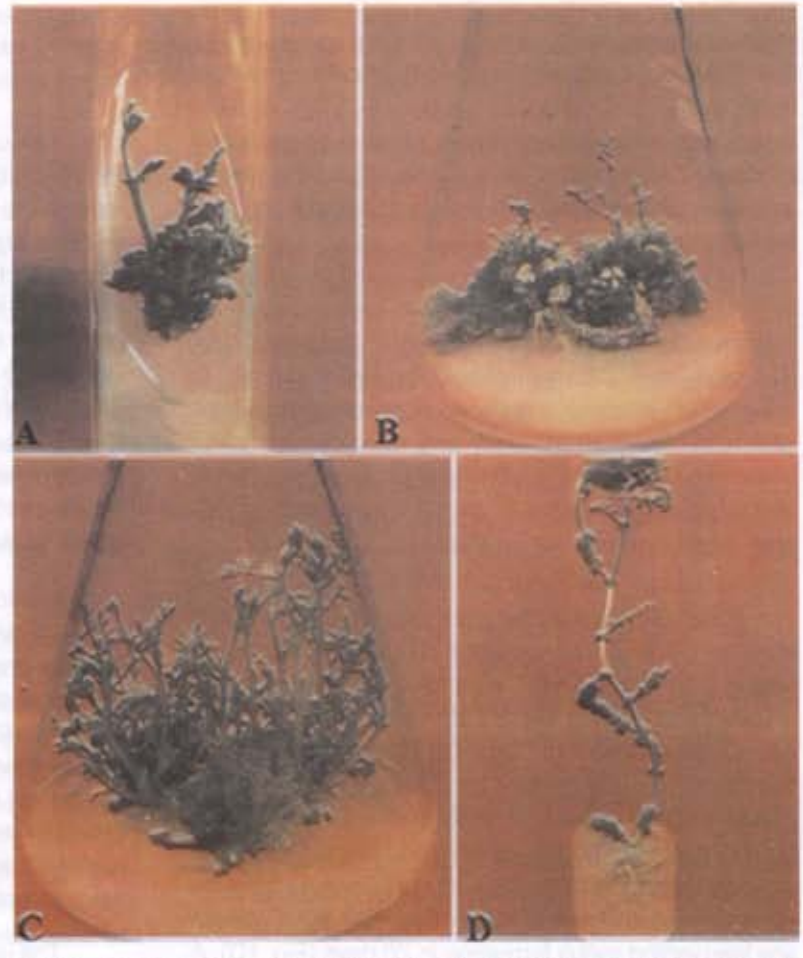

Fig.1A. Initiation and proliferation of callus from shoot tip explant. IB. Proliferation of shoot buds and shoots development. 1C. Regeneration of multiple shoots with vigorous growth. ID. Induction of roots in isolated shoot in rooting media.

Acknowledgments

The author is thankful to Dr. H.C. Chaturvedi for his useful suggestions and to the Director, National Botanical Research Institute, Lucknow, for the facilities provided. Award of Biotechnology National Associateship by the Department of Biotechnology, Govt. of India, New Delhi to the author is also gratefully acknowledged.

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